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STUDIES ON THE METABOLISM OF METHANOL AND FORMALDEHYDE IN THE ANIMAL ORGANISM

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Martti Koivusalo

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INTRODUCTION

Methanol (CH_3OH) is the lowest member of the series of primary saturated aliphatic alcohols. Its biological behaviour is markedly different from that of the next homologue, ethanol. Although the acute toxicity of methanol is apparently lower than that of ethanol, methanol has a specific, highly toxic late effect, i.e., its subacute and chronic toxicities are high. Very typical for methanol poisoning in man is the transitory or permanent blindness, amblyopia, which already may follow the ingestion of small amounts of methanol. Since methanol poisonings are rather usual especially during times of war and shortages, considerable attention has been devoted to methanol especially in toxicological and clinical literature. The significance of methanol, and especially that of its metabolites formaldehyde and formic acid, does not, however, seem to be confined to the field of toxicology. Certain nutrients, as, for instance, fruits, contain much pectin from which methanol is liberated in the organism. The tremendous advance during recent years in our knowledge of the metabolism of the so called one-carbon compounds has demonstrated that formaldehyde and formic acid or their derivatives are of a great significance in normal intermediary metabolism. The organism is capable of also using methanol for the formation of many important compounds.

In the following chapter a general survey is given of the literature concerning the metabolism of methanol and formaldehyde in the animal organism. Clinical and pathological aspects have not been discussed and the reader is referred in this respect to the monographs and papers of RØE (1943, 1946, 1955) and ORTHNER (1955). More detailed surveys of the rapidly expanding literature on the metabolism of one-carbon compounds can be found

in the reviews of, e.g., WELCH and NICHOL (1952), CANTONI (1952, 1956), ARNSTEIN (1955) and BACH (1955).

It was the object of the experiments described in the present report to study the ability of the animal organism to utilize methanol and formaldehyde metabolically and to investigate various factors associated with these reactions.

REVIEW OF THE LITERATURE

METABOLISM OF METHANOL IN THE ANIMAL ORGANISM

Oxidation of Methanol

The earliest investigations on the metabolism of methanol in the animal organism were made towards the end of the past century by Pohl (1893), who demonstrated that methanol is converted in the organism to formic acid. He found that when methanol was administered to dogs, the urinary excretion of formic acid was markedly increased for several days. The maximum increase occurred during the second to fourth days after the administration of methanol, when the toxic symptoms also were most severe. The excretion of formic acid in the urine was found to be increased also in humans to whom small amounts of methanol were administered. Pohl demonstrated further the formation of formic acid from methanol in experiments *in vitro* with dog liver preparations.

Pohl's results have since been confirmed by several investigators. The increased urinary excretion of formic acid after the intake of methanol has been found in dogs (Bongers 1895, Asser 1914, Harrop and Benedict 1920, Leo 1927, Bastrup 1947, and Lund 1948 b), cats (Rost and Braun 1926), and humans (Autenrieth 1922, Kendal and Ramanathan 1953). The determination of the formic acid concentration in the urine in cases of suspected methanol poisoning has also been used as a diagnostic aid (Klauer 1938, Müller 1939). There apparently exist rather great differences between different species of animals in the relative amounts of formic acid excreted in the urine after the intake of methanol. Although increased values have been found in rabbits they have not been present in all cases (Asser 1914, Pohl 1922, Rost and Braun 1926). In comparison to humans and dogs, the increase in the excretion of formic acid by methanol-treated rabbits is much lower. Only 1 per cent or less of the administered methanol can be recovered from the urine as formic acid in rabbits; in dogs the value is 14—20 per cent (Bastrup 1947, Lund 1948 a, b, c). This difference is apparently due to the greater capacity of rabbits to metabolize formic acid (Bastrup 1947, Lund 1948 a, b).

These findings have clearly demonstrated that methanol is at least partly converted to formic acid in the organism. The most simple and natural mechanism for this conversion is, of course, oxidation, with formaldehyde as intermediate. Already POHL (1893) tried to demonstrate formaldehyde in the urine and tissues of dogs poisoned with methanol, but with negative results. Despite several attempts to detect formaldehyde in cases of methanol poisoning, it was not until 1931 that this was successful for the first time. KEESER (1931 a) then demonstrated by qualitative tests the presence of formaldehyde in the cerebrospinal fluid, vitreous humour of the eye and peritoneal fluid of rabbits which had been poisoned with methanol.

Only very few studies have been made on the formation of formaldehyde from methanol *in vitro*. A positive qualitative test for formaldehyde was obtained when fresh vitreous humour of the ox was incubated with methanol (KEESER 1931 b). In further experiments KEESER and VINCKE (1940) and HELLWIG (1940) demonstrated qualitatively the formation of formaldehyde during the incubation of methanol with ground horse liver.

Formaldehyde has also been detected as its dimedone derivative in rat liver homogenates incubated with methanol (MACKENZIE 1950). The enzyme system responsible for the conversion of methanol to formaldehyde is located in the supernatant fraction of the rat liver homogenate and no activity has been found in the sediment fraction (MACKENZIE, JOHNSTON and FRISELL 1952, 1953).

The oxidation of carbon-labelled methanol to carbon dioxide in the intact rat occurs at a constant rate of 25 mg per kg per hour (BARTLETT 1950 a). In similar experiments it had been shown earlier that ethanol is oxidized at a constant rate of 175 mg per kg per hour (BARTLETT and BARNET 1949). Ethanol is thus oxidized seven times as rapidly as methanol. This constant rate indicates that the oxidation to carbon dioxide in the rat is not dependent to any great degree on the blood alcohol level. Of the ingested methanol 60 per cent was directly oxidized to carbon dioxide, 14 per cent was eliminated unchanged in the expired air, and 3 per cent was recovered as such in the urine. The elimination of unchanged methanol in the expired air was dependent on the blood methanol concentration. The tissues were able to fix appreciable quantities of methanol carbon. Most active in this respect were the liver, kidney and intestine. A constant rate of methanol oxidation to carbon dioxide has also been found in mice, in which the oxidation rate was one and a half times that in rat, i.e., 37 mg per kg per hour (HEVESY 1953).

Methanol is oxidized to carbon dioxide also in rat tissue slices (BARTLETT 1950 a). Liver slices were most active, and the relative capacities of other tissues were: liver, 100; kidney, 34; intestine, 8; heart, 1; diaphragm, 1; brain, 0. Considerable amounts of methanol carbon were fixed in the slices, this being in the liver 25 per cent of the amount oxidized to carbon dioxide.

Our knowledge of the enzymic details of the oxidation of the methanol

to formaldehyde is very meagre. Two enzymes are known to exist in the animal organism which may be implicated in the oxidation of methanol to formaldehyde *in vivo*, namely alcohol dehydrogenase and catalase.

Alcohol dehydrogenase catalyses the following reaction:

$$\text{ethanol} + \text{DPN} \rightleftarrows \text{acetaldehyde} + \text{DPNH} + \text{H}^+$$

This enzyme is widely distributed in plant and animal tissues. Best known are the enzymes from yeast and horse liver. The first soluble alcohol dehydrogenase preparation from animal tissues was obtained by BATTELLI and STERN (1909, 1910 a, b). The dependence of animal alcohol dehydrogenase on diphosphopyridine nucleotide was demonstrated by QUIBELL (1938). A slight activity also in the presence of triphosphopyridine nucleotide was found later (PULLMAN, COLOWICK and KAPLAN 1952). Partially purified preparations have been obtained from horse liver by water extraction and precipitation with acetone (LUTWAK-MANN 1938).

Crystalline alcohol dehydrogenase was first prepared from yeast by NEGELEIN and WULFF (1936, 1937). It is a typical sulphydryl enzyme exhibiting marked sensitivity toward iodoacetate (e.g., DIXON 1937) and other sulphydryl reagents (BARRON and SINGER 1945).

Animal alcohol dehydrogenase has been isolated in crystalline form from horse liver (BONNICHSEN and WASSÉN 1948, BONNICHSEN 1950). The liver enzyme is in many respects different from the yeast enzyme: it contains less aromatic amino acids (BONNICHSEN 1950, NEGELEIN and WULFF 1937), is not inhibited by iodoacetate (LUTWAK-MANN 1938), and has a broader substrate specificity. The molecular weight of the liver enzyme is lower than that of the yeast enzyme (THEORELL and BONNICHSEN 1951). The liver enzyme oxidizes also vitamin A to retinene, which may also be of physiological significance (BLISS 1951, HUBBARD and WALD 1951). The activity of the crystalline horse liver alcohol dehydrogenase is remarkably low, the maximal turnover number when ethanol is used as substrate having been reported to be only 140 mol. DPN/mol. ADH × min. (THEORELL and BONNICHSEN 1951). Horse liver has been found to contain as much as 0.1 per cent alcohol dehydrogenase (BONNICHSEN 1950). Purified preparations from rat liver had only a slight alcohol dehydrogenase activity and those from guinea pig liver had no measurable activity (NYBERG, SCHUBERTH and ÄNGGÅRD 1953).

The kinetics of the horse liver alcohol dehydrogenase have been extensively studied by THEORELL and his associates. The enzymic kinetics of liver alcohol dehydrogenase have been found to correspond very well to the rectilinear course of the oxidation of ethanol *in vivo*, and there is quite convincing evidence that alcohol dehydrogenase is the enzyme essentially responsible for the physiological oxidation of ethanol (THEORELL and BONNICHSEN 1951).

The oxidation of methanol by liver alcohol dehydrogenase, on the other hand, is not yet settled. Of the earlier authors, POHL (1893) and BATTELLI

and STERN (1910 a, b) already found that liver preparations were able to oxidize methanol to formic acid. The oxidation of methanol was found to be more labile than the oxidation of ethanol by the same preparations (BATTELLI and STERN 1910 a, b). The more purified alcohol dehydrogenase preparations of LUTWAK-MANN (1938) from horse liver also oxidized methanol, although very slowly. ZATMAN (1946) used similar preparations and found methanol to be oxidized at one-ninth of the rate of ethanol oxidation in the presence of diphosphopyridine nucleotide. The crystalline horse liver alcohol dehydrogenase does not, however, react with methanol and diphosphopyridine nucleotide to any measurable extent (BONNICHSEN and THEORELL 1951, THEORELL and BONNICHSEN 1951). It has been demonstrated, however, that the same enzyme preparation catalyses the reverse reaction, namely the reaction of formaldehyde with reduced diphosphopyridine nucleotide. This reaction, however, is much slower than the corresponding reaction of alcohol dehydrogenase with acetaldehyde and reduced diphosphopyridine nucleotide (THEORELL and CHANCE 1951). As a possible explanation for the failure of the crystalline enzyme to oxidize methanol THEORELL and BONNICHSEN suggested (1951) that the oxidation of methanol may require some additional catalyst besides alcohol dehydrogenase and diphosphopyridine nucleotide. Yeast alcohol dehydrogenase in high concentrations is capable of oxidizing methanol, although slowly (BARRON and LEVINE 1952).

KEILIN and HARTREE (1936, 1945) have demonstrated that *catalase* can oxidize ethanol and methanol in *in vitro* systems which consist in a primary oxidizing system generating hydrogen peroxide, and in alcohol and catalase. The hydrogen peroxide formed reacts with the alcohol, oxidizing it to the corresponding aldehyde. The latter reaction is catalyzed by the catalase, which functions peroxidatically in these »coupled oxidations».

The primary oxidizing system is formed by oxidizing enzymes such as xanthine oxidase, uricase, d-amino acid oxidase, tyraminase, glucose oxidase and others, which catalyse the oxidation of their respective substrates by molecular oxygen, reducing the latter to hydrogen peroxide.

Since the discovery of catalase it has been held that the main function of catalase is protective and consists in the decomposition of hydrogen peroxide into oxygen and water according to the equation

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

However, KEILIN and HARTREE (1945) consider the aforementioned coupled oxidations a more likely biological function of catalase.

In a system composed of xanthine oxidase, aldehyde, catalase and alcohol, the hydrogen peroxide formed in the primary oxidation of aldehyde to acid is utilized by catalase for the oxidation of alcohol to aldehyde (KEILIN and HARTREE 1945). Thus the product of the secondary oxidation supplies the substrate for the primary oxidation system and this »cyclic oxidation» reaction will proceed as long as alcohol is present and the en-

zymes are active. The reactions in the case of methanol and formaldehyde can be written as follows:

$$HCHO + H_2O + O_2 \rightarrow HCOOH + H_2O_2$$
$$H_2O_2 + CH_3OH \rightarrow HCHO + 2\ H_2O$$

CHANCE (1947) has demonstrated that catalase forms very rapidly an intermediate compound in the presence of hydrogen peroxide and this intermediate compound then reacts with ethanol or methanol, yielding the corresponding aldehyde. He has calculated that the peroxidatic activity of the intermediate catalase-hydrogen peroxide compound is adequate to account for the results of the experiments of AGNER and BELFRAGE (1947) on the kinetics of the disappearance of methanol from the blood in rabbits. He suggested, therefore, that the oxidation of methanol *in vivo* may be ascribed to the peroxidatic functioning of catalase.

JACOBSEN (1952 a, b) also considered catalase a very possible mechanism for the oxidation of methanol *in vivo*. The rôle of catalase in the oxidation of ethanol, on the other hand, cannot be great, for the enzymic kinetics of catalase action (CHANCE 1947) are not compatible with the rectilinear elimination of ethanol from blood. It has been suggested, however, that the deviations from rectilinearity in the blood ethanol elimination curves which have been found by some authors may be due to the participation of catalase in addition to alcohol dehydrogenase in the oxidation of ethanol (JACOBSEN 1952 a, b). The aforementioned results of THEORELL and BONNICHSEN (1951) which show no methanol activity for crystalline horse liver alcohol dehydrogenase also support the possible participation of catalase in the oxidation of methanol *in vivo*.

Despite the above evidence which speaks for the oxidation of methanol *in vivo* by catalase, there are also many opposite observations. The inhibitory effect of ethanol on the oxidation of methanol, which has been observed both *in vivo* and *in vitro* (cf. page 17), is not likely in the case of catalase unless ethanol inhibits the primary oxidation systems which *in vivo* provide the catalase with hydrogen peroxide (CHANCE 1947). The different oxidation rates of methanol and ethanol *in vivo* have also been presented as evidence against catalase (BARTLETT 1952). In the *in vitro* systems the rates of reaction of catalase with ethanol and methanol have been of the same magnitude (KEILIN and HARTREE 1945, CHANCE 1947), and in view of the similar tissue penetrability of these two alcohols they should both be oxidized at the same rate also *in vivo* by catalase. A skeptical view towards the oxidation of alcohols by catalase *in vivo* has been taken also by LEMBERG and LEGGE (1949) and KENDAL and RAMANATHAN (1953).

The following step in the oxidation of methanol is the *oxidation of formaldehyde* to formic acid, which will be discussed in the following section.

The enzymic details of the *oxidation of formic acid* to carbon dioxide are also rather poorly known. Formate is readily metabolized in the intact

animal (e.g., WEINHOUSE and FRIEDMANN 1952) as well as in homogenates of various tissues (NAKADA and WEINHOUSE 1953). MATTHEWS and VENNESLAND (1950) demonstrated in rat liver the presence of an adenosine triphosphate-dependent formicodehydrogenase, which oxidizes formic acid to carbon dioxide and water. Formate can also be oxidized *in vitro* by a catalase-hydrogen peroxide complex (CHANCE 1949, 1950). NAKADA and WEINHOUSE (1953) presented evidence that the oxidation of formate *in vivo* was due to catalase and suggested that the above mentioned formicodehydrogenase was actually a coupled oxidation phenomenon resulting from the combined action of xanthine dehydrogenase and catalase.

METHANOL AS A SOURCE OF LABILE METHYL GROUPS

The present concept of transmethylation reactions is largely based on the work of DU VIGNEAUD and his associates, although the existence of some transmethylation reactions was known already earlier (cf. CHALLENGER and HIGGINBOTTOM 1935). Nutritional experiments with rats demonstrated that to sustain growth the diet must contain compounds which can act as labile methyl group donors. It was concluded that the organism cannot synthetize methyl groups (DU VIGNEAUD, CHANDLER, MOYER and KEPPEL 1939). However, some animals were able exceptionally to sustain growth without exogenous labile methyl groups and it has later been shown that in the presence of sufficient amounts of folic acid and vitamin B_{12} the organism is capable of forming labile methyl groups *de novo* from precursors like formate and formaldehyde or from compounds which in the metabolism give rise to formate or formaldehyde (e.g., BENNETT 1949, 1950, SAKAMI and WELCH 1950, DU VIGNEAUD, RESSLER, RACHELE, REYMIRS and LUCKEY 1951). For a detailed discussion of the metabolic reactions of one-carbon compounds the reader is referred to the several recent reviews by, e.g., WELCH and NICHOL (1952), CANTONI (1952, 1956), ARNSTEIN (1955), BACH (1955) and others.

DU VIGNEAUD and VERLY (1950) and ARNSTEIN (1950 a, b) demonstrated in isotopic tracer experiments *in vivo* that methanol can be utilized by the rat in the formation of biologically labile methyl groups. In earlier nutritional experiments methanol had failed to support the growth of rats on a diet which was free of biologically labile methyl groups and which contained homocystine (DU VIGNEAUD *et al.* 1939). The methyl group of methanol is apparently not utilized at least to any great extent directly in transmethylation reactions (DU VIGNEAUD, VERLY, WILSON, RACHELE, RESSLER and KINNEY 1951). This was demonstrated in experiments where rats were administered methanol which was doubly labelled with isotopic carbon and deuterium in the methyl group. The ratio $D:C^{14}$ in the methyl groups of choline isolated from the carcass was found to be lower than in the administered methanol; this indicated oxidation and subsequent reduction to the labile methyl group in the biosynthesis of the latter. In

transmethylation reactions the methyl group is transferred with its three hydrogen atoms (KELLER, RACHELE and DU VIGNEAUD 1949).

Folic acid and vitamin B_{12} are apparently both necessary in these reactions of methanol, which is well in accordance with the recent concept of the functions of these factors in the metabolism of one-carbon compounds. It is generally regarded that folic acid functions both in transmethylation and in methyl group synthesis *de novo*, and vitamin B_{12} only in the latter (cf. ARNSTEIN 1955).

Folic acid has been found to increase the incorporation of C^{14} from methanol into the choline methyl groups in rats which were deficient in folic acid (VERLY, KINNEY, and DU VIGNEAUD 1952, VERLY, WILSON, KINNEY and RACHELE 1954, VERLY and CATHEY 1955). Also the folic acid-derivative leucovorin (synthetic citrovorum factor, 5-formyl- 5: 6: 7: 8-tetrahydropteroylglutamic acid) is active in this respect (VERLY *et al.* 1952). On the other hand, the impairment in creatine metabolism in folic acid-deficient mice has been reported to be partially restored by the administration of methanol, which speaks against the direct participation of folic acid in the methyl group synthesis from administered methanol (FATTERPAKER, MARFATIA and SREENIVASAN 1952). Rats which were deficient in vitamin B_{12} incorporated less isotope from methanol into the choline methyl groups than the control rats to which was given vitamin B_{12} (VERLY and CATHEY 1955). If the rats were deficient in both vitamin B_{12} and folic acid this incorporation was less than in rats which were deficient only in vitamin B_{12}.

ELIMINATION OF METHANOL FROM BLOOD

Although numerous studies have been carried out concerning the elimination of ethanol from the blood, only few similar studies have been made with methanol, and the results, furthermore, are rather conflicting.

NICLOUX and PLACET (1912) studied the elimination of methanol in dogs and rabbits after peroral administration. In the experiments with dogs the doses were 3 and 5 ml of methanol per kg. Traces of methanol were still present in the blood after 120 and 102 hours, respectively. The elimination rate from blood was in dogs 0.0005—0.0008 mg per ml per minute. When 5 ml of methanol per kg was given to a rabbit the blood was free from methanol after 52 hours, and the elimination rate was 0.0012 mg per ml per minute. The elimination curves were practically linear both in dogs and rabbits. Less methanol was recovered from the urine and from the air in the metabolic chamber in the experiments with rabbits than in those with dogs, which together with the greater elimination rate from blood may be taken as an indication of greater capacity of rabbits to metabolize methanol.

Several studies have been made using WIDMARK's (1922) method for blood ethanol determination as adapted by BILDSTEN for the determination

of methanol (BILDSTEN 1924). The elimination of intravenously administered methanol from the blood of rabbits was studied by BILDSTEN (1924) and WIDMARK and BILDSTEN (1924). The blood methanol concentration curves were determined in six rabbits given about 0.8 g methanol per kg. The elimination rate varied from 0.00057 to 0.00117 mg per g per minute, averaging 0.00076. In these experiments the elimination of methanol from the blood occurred at a constant rate which was not dependent on the concentration of methanol in the blood. In nearly all of the elimination curves there was found a temporary increase in the methanol concentration 4—6 hours after the administration. The cause of this increase is not known. BILDSTEN (1924) regarded it as quite possible that this increase could be ascribed to some substance other than methanol, which accumulated temporarily during the metabolism of methanol and was determined as methanol by the dichromate method used. BERNHARD and GOLDBERG (1933) administered to rabbits 0.56 to 2.10 g methanol per kg intravenously. The normal elimination rate was found to be on an average 0.00067 mg per g per minute. In general the elimination curves were linear and the temporary increase 4—6 hours after administration was seen in most curves. The elimination rate was found to vary considerably in the same animal at different times. When the ventilation rate of the rabbits was increased to about 2—3-fold by administration of carbon dioxide, the elimination rate of methanol increased on an average by 27 per cent. The elimination rate of methanol in dogs was studied by NEYMARK (1936) and NEYMARK and WIDMARK (1936) and was found to be 0.00018 mg per g per minute and independent of the dosage. The temporary increase seen in the experiments with rabbits was not noted in these elimination curves.

HAGGARD and GREENBERG (1939), differing from the above, concluded that in rats and dogs the rate of elimination is proportional to the concentration of methanol in the body. In their opinion most of the ingested methanol is excreted in the expired air as a result of simple diffusion and therefore the elimination rate is directly proportional to the concentration of methanol in the blood, and the blood concentration curve is consequently exponential. In experiments with rats, they recovered in the expired air an average of 76 per cent of the methanol which had disappeared from the body. However, only 6 per cent of the total dose had been eliminated during the experiment. Carbon dioxide and 2: 4-dinitrophenol increased the elimination of methanol presumably by increasing pulmonary ventilation. Exponential methanol elimination curves for dogs were also presented by these authors.

The elimination rate of methanol from blood was correlated to the blood concentration of methanol also in the experiments with rabbits conducted by AGNER and BELFRAGE (1947). The methanol determinations were made by the specific chromotropic acid method. Methanol was given both orally and intravenously in doses of 1.7 to 2.2 g per kg. At a blood methanol concentration higher than about 1 mg per ml the rate of elimination was about 0.0017 mg per ml per minute, and at lower concentrations about

0.0006 mg per ml per minute. The temporary increase in the blood methanol concentration seen in the experiments of BILDSTEN (1924), WIDMARK and BILDSTEN (1924) and BERNHARD and GOLDBERG (1933) was not found.

The elimination of small amounts of methanol has also been studied in human subjects (LEAF and ZATMAN 1952). The decline in the concentration of methanol in the body was determined in these experiments by following the concentration of methanol in the urine. The reliability of this procedure was confirmed by experiments with cats and human subjects, in which a constant ratio was found between the methanol concentration in the blood and that in the contemporaneously secreted urine. Variable doses of 2.5—7.0 ml methanol were employed. The rate of elimination was at all times found to be proportional to the concentration of methanol in the body. Only a very small fraction of the ingested methanol — about 2 per cent — was eliminated as such by the respiratory and urinary routes.

EFFECT OF ETHANOL ON THE METABOLISM OF METHANOL

The effect of ethanol on the metabolism of methanol has in addition to its theoretical significance also a considerable clinical and toxicological importance since most persons who drink methanol consume it with ethanol, as in various denaturated spirits. Ethanol has also been used in the therapy of methanol poisoning.

Most statements in the older clinical literature concerning the effect of ethanol on methanol poisoning are purely speculative. Most of the authors were of the opinion that ethanol increases the toxicity of methanol (BÜRGER 1912, GETTLER and GEORGE 1912, FOERSTER 1912, PANTALEONI 1927, NEIDING, GOLDENBERG and BLANK 1932); however, already early in the present century ethanol was also advocated as stimulant in the therapy of methanol poisoning (WOOD and BULLER 1904).

In extensive clinical studies RØE (1943, 1946) observed that in certain cases of methanol poisoning in which ethanol also had been consumed the poisoning was less severe than when only methanol was ingested and he regarded the principal cause of the great tolerance to methanol shown by some individuals to be the consumption of ethanol just before or during the methanol poisoning. In cases of uncomplicated methanol poisoning it was never noted that a person drinking less methanol than a companion suffered more severely than the latter, as might be expected to happen if individual predisposition were a factor of decisive importance, as was the opinion earlier. Ethanol, when drunk at the same time as methanol, seemed to prolong the latent period by the same interval as that required for the oxidation of ethanol. Ethanol taken after methanol but before the onset of symptoms was able to prolong the latent period by a longer interval than that required for its oxidation, and severe symptoms also quickly vanished after the consumption of ethanol late in the course of the poisoning. RØE explains the effect of ethanol by its greater surface activity,

which enables it to prevent the adsorption of methanol to the oxidative enzymes and consequently its conversion to formic acid. On the basis of these observations he also advocates the use of ethanol in addition to bicarbonate in the therapy of methanol poisoning.

ORTHNER (1950) takes an entirely opposite standpoint in regard to the effect of ethanol. He states that ethanol taken simultaneously with methanol dilates the blood vessels and raises the permeability, thus increasing the special toxic effects of the metabolic products of methanol.

In white mice the median lethal dose (LD_{50}) for a single intraperitoneal injection of methanol is significantly decreased by ethanol (GILGER, POTTS and JOHNSON 1952). Similar results have also been obtained in experiments with guinea pigs (MOESCHLIN and GARSON 1955). However, these experiments have dealt mainly with the acute toxicity of methanol, which is due to its narcotic effects and is much smaller than that of ethanol.

According to von FELLENBERG (1917), the low urinary excretion of methanol found after pectin-rich food is considerably increased when ethanol is also consumed. He suggested that the oxidation of methanol becomes more difficult when the organism must simultaneously oxidize large amounts of ethanol.

ASSER (1914) found in experiments on dogs and rabbits that when he gave ethanol simultaneously with methanol the increased excretion of formic acid in the urine decreased appreciably. Acetone and amyl alcohol had a similar effect. As the most appropriate explanation he considered the possible increase in the oxidation of the formic acid due to an increased permeability of the cells. This decrease in the urinary excretion of formic acid after the simultaneous intake of ethanol and methanol has since been confirmed in experiments with dogs and rabbits (BASTRUP 1947) as well as in man (KENDAL and RAMANATHAN 1953). In the latter it was found that when the dosage of methanol was 4 ml, about 10 ml of ethanol per hour were required to suppress totally the increased urinary excretion of formic acid.

Human experiments were also conducted by LEAF and ZATMAN (1952). In these experiments ethanol was taken at various times during the elimination of a fixed dose, 4 ml, of methanol. A single dose of 15 ml ethanol taken simultaneously caused a marked elevation in the peak concentration of methanol attained in the urine. When ethanol was taken after methanol it effectively arrested the decline in the urinary methanol concentration. After about two hours the decline occurred at the original rate. In all of their experiments it was seen that when ethanol was administered during the elimination of methanol, the decline in the methanol concentration in the urine was very slow. The reason for this effect of ethanol the authors consider to be an inhibition of the oxidation of methanol by alcohol dehydrogenase.

The oxidation of isotope-labelled methanol to carbon dioxide in rats *in vivo* is strongly inhibited by the simultaneous administration of ethanol

(Bartlett 1950 b). When a sufficient amount of ethanol was given the oxidation of methanol ceased totally, and after the apparent oxidation of ethanol that of methanol continued at the same rate as before the administration of ethanol. The inhibitory action of ethanol on the metabolism of methanol was apparent also in the experiments of Agner and Belfrage (1946), in which they administered simultaneously methanol and ethanol to two rabbits. The elimination of methanol from blood was distinctly retarded by ethanol.

We have very little knowledge of the effects of ethanol on the metabolism of methanol in *in vitro* systems. Zatman (1946) mentioned in a brief note the results of experiments on the effect of ethanol on the oxidation of methanol by the crude alcohol dehydrogenase from horse liver prepared according to Lutwak-Mann (1938). This preparation was found to oxidize methanol at about one-ninth of the rate for the oxidation of ethanol. When mixtures of methanol and ethanol were used as substrate the oxidation of methanol was inhibited by ethanol, as judged from the decreased amount of formaldehyde formed. When the alcohols were present in equimolar amounts no formaldehyde was formed and the inhibition was still distinct when the molar ratio of ethanol and methanol was only 1 : 16.

Added ethanol depressed the oxidation of isotope-labelled methanol by rat liver slices as a linear function of the logarithm of the ethanol concentration (Bartlett 1950 b). In these experiments a 72 per cent inhibition of the carbon dioxide formation from methanol was obtained when both ethanol and methanol were present in a concentration of 0.01 M.

Pathogenesis of Methanol Poisoning

Since the end of the past century, when the first cases of human methanol poisoning were described, the cause of the specific toxicity of methanol has been much debated. Although many different hypotheses, some purely speculative and others based on clinical or experimental observations, have been presented, we do not as yet exactly know how the specific toxic action of methanol is brought about.

It was earlier a rather generally accepted opinion that methanol itself is not very toxic and that the toxicity depends on various impurities in the methanol (fusel oil) (Stadelmann and Magnus-Levy 1912, Igersheimer and Verzár 1913, Rostedt 1922, Hämäläinen and Teräskeli 1928, and others). This theory, however, had to be abandoned since it was repeatedly shown that also chemically pure, synthetically produced methanol was the cause of numerous poisonings (e.g., Reif 1923, Alder, Buschke and Gordonoff 1938). The acute toxicity of methanol is lower than that of ethanol, and the so called Richardson's law that the toxicity of alcohols increases in the homologous series in the ratio $1 : 3 : 3^2 \ldots$ was shown to be valid also in the case of methanol and ethanol (Führer 1905,

WEESE 1926). However, some authors have considered methanol as such to be responsible for its specific toxicity (REIF 1926, DINSLAGE and WINDHAUSEN 1926). Already POHL (1893) had shown that administered methanol remains in the organism for a long time and this cumulation was regarded as the basis of the toxicity by, e.g., SIMON (1933) and EGG (1926).

Already quite early the oxidation products of methanol, — formaldehyde and formic acid — were held as responsible for its toxicity. POHL (1893) had demonstrated that the administration of methanol increased the urinary excretion of formic acid, and many authors attributed the poisoning to formic acid (e.g., HARNACK 1912, LEO 1925). Still more ascribed it to formaldehyde (RABINOWITCH 1922, BRÜCKNER 1924, and others) although attempts to demonstrate formaldehyde in blood, urine and tissues in cases of methanol poisoning had been unsuccessful (e.g. POHL 1893, GETTLER and GEORGE 1918). The great reactivity of formaldehyde was regarded as the reason for this failure to demonstrate it. The occurrence of formaldehyde in the organism in methanol poisoning was demonstrated for the first time by KEESER (1931a). He obtained positive tests for formaldehyde in the cerebrospinal fluid, vitreous humour of the eye and peritoneal fluid of rabbits which were poisoned with methanol. Most of the later authors were also of the opinion that the specific toxicity of methanol is due to the slow formation of formaldehyde inside the cells (FLURY and WIRTH 1936, HAILE 1949, ORTHNER 1950). The combination of formic acid with the iron in haemoglobin and in oxidative enzymes of the cells, and the consequent hypoxia are according to RØE (1943, 1946, 1955) the primary factors in the toxicity of methanol in man. KENDAL and RAMANATHAN (1953) suggested the possibility that after primary conversion of part of the methanol into formaldehyde a secondary conversion of the latter into methyl formate may occur by a semiacetal dehydrogenase mechanism which they had observed *in vitro*. The preferential fat-solubility of the ester would then result in the localization and specific effects of methanol poisoning.

The acidosis which is repeatedly observed in human cases of methanol poisoning apparently plays an important rôle in the development of the typical manifestations of the poisoning (HARROP and BENEDICT 1920, RABINOWITCH 1922, RØE 1943, 1946). In experimental animals only slight acidosis has been observed (HASKELL, HILLEMAN and GARDNER 1921, LOEWY and MÜNZER 1923, LEO 1925). This acidosis has often been regarded as directly due to formic acid but the amounts of formic acid formed are not sufficient to account for the marked diminution of the blood alkali reserve often seen (EGG 1927, RØE 1946). A considerable increase has been observed in lactic acid in the blood (RØE 1946) and the urine (HARROP and BENEDICT 1920) and this may in part be responsible for the acidosis. The methylenation of amino groups in amino acids and proteins has also been suggested as a factor in the development of the acidosis (RABINOWITCH 1922, ORTHNER 1950).

METABOLISM OF FORMALDEHYDE IN THE ANIMAL ORGANISM

The occurrence of formaldehyde as a normal metabolite in the animal organism was denied for a long time and only a toxicological rôle was given to it. During the past few years, however, when our knowledge of the metabolism of one-carbon compounds has greatly advanced, formaldehyde has also gained new significance. It has now been shown to be a possible intermediate in many important biological reactions.

The reactions of formaldehyde in the intermediary metabolism can be divided into four groups, i.e., reactions where formaldehyde is formed, directly oxidative reactions, various condensation reactions, and reactions which link it to the metabolism of the one-carbon group.

Formation of Formaldehyde

The formation of formaldehyde from methanol has been discussed in the earlier chapter. However, formaldehyde may also be formed from other, in some cases more physiological compounds than methanol.

When various *N*-methylated compounds are demethylated by animal tissue preparations, it has been found that formaldehyde is formed from the methyl group. Handler, Bernheim and Klein (1941) found that broken cell preparations of rabbit, rat and guinea pig liver are able to oxidatively demethylate sarcosine to glycine. They obtained strongly positive qualitative reactions for formaldehyde in the solution of oxidized sarcosine.

Mackenzie and Du Vigneaud (1949) and Mackenzie (1950) studied further the biological oxidation of the methyl group of sarcosine in rat liver homogenates. They obtained radioactive formaldehyde as an oxidation product of sarcosine labelled in the methyl group with C^{14}. Formaldehyde was also isolated and identified by them in the form of dimedone derivative.

All the methyl groups which are known to be oxidized to formate and carbon dioxide do not apparently produce formaldehyde with the same facility during this reaction. Formaldehyde was obtained from sarcosine, dimethylaminoethanol, dimethylglycine and methanol incubated with rat liver preparations, but not from methionine, betaine, choline, glycine, serine, monomethylaminoethanol or aminoethanol by Mackenzie, Johnston and Frisell (1953). The degradation of serine to glycine and formaldehyde, on the other hand, has been demonstrated in various liver preparations by Vilenkina (1949, 1952), Blakley (1954 a, b) and Alexander and Greenberg (1955). Siekevitz and Greenberg (1950) obtained labelled formaldehyde from the methyl groups of methionine and choline and from the α-carbon of glycine and β-carbon of serine after incubation

with rat liver slices. The apparent discrepancies in these results are probably due to differences in the tissue preparations and experimental techniques used.

YOSIDA and FUKUYAMA (1941, 1944) investigated the demethylation of *N*-methyl-L-tryptophane and found that preparations from kidney and liver of rabbit, guinea pig, pigeon and hen oxidize it to L-tryptophane and formaldehyde by direct demethylation, which they ascribed to the presence of a demethylase, probably a flavoprotein (YOSIDA 1944). Further investigations on the rabbit kidney demethylase have been reported by LING and TUNG (1948), TUNG and COHEN (1952) and MORITANI, TUNG, FUJI, MITO, IZUMIYA, KENMOCHI and HIROHATA (1954). The demethylase is capable of oxidizing only *N*-methyl-L-amino acids to formaldehyde and the corresponding L-amino acid. It is different from sarcosine oxidase, although their action on α-methylamino acids is analogous. Further evidence that demethylase is a flavine-adenine dinucleotide enzyme was also obtained.

Certain *N*-methylated aminoazo dyes, which are hepatic carcinogens, have also been shown to be oxidatively demethylated in fortified rat liver homogenates to form stoichiometric amounts of formaldehyde and primary aminoazo dye (MUELLER, and MILLER 1951, 1953). These methyl groups can also partly be transferred to choline and serine in *in vitro* experiments (MILLER, PLESCIA, MILLER and HEIDELBERGER 1952).

OXIDATION OF FORMALDEHYDE

There is very little information concerning the direct oxidation of formaldehyde in animal tissue preparations. Rat liver mitochondria are able to oxidize formaldehyde in small concentrations, high formaldehyde concentrations being inhibitory (WALKENSTEIN and WEINHOUSE 1953). BERNHEIM (1950) found that added formaldehyde inhibits the endogenous oxygen uptake of unwashed liver homogenates for a while, after which the formaldehyde is apparently oxidized, although rather slowly. However, if aminoguanidine is added with the formaldehyde the latter is immediately oxidized without initial inhibition. In washed rat liver preparations formaldehyde is oxidized only if aminoguanidine is also present. The oxidation of the formed formaldehyde-aminoguanidine complex is inhibited by methyl-bis-(β-chlorethyl)-amine (MILLER 1953). This substance inhibits also choline oxidation to the same degree and it has also been suggested that these oxidations may be mediated by the same enzyme or may require the same or related cofactors (MILLER 1953). Aminopterin treatment also causes a marked inhibition of the oxidation of both choline and the formaldehyde-aminoguanidine complex in mouse liver homogenates (MILLER 1954). No further details are known concerning this remarkable action of aminoguanidine and its physiological significance is not clear. Aminoguanidine decreases the acute toxicity of formaldehyde to mice, presumably by increasing its oxidation (ZAUDER 1950). However, this effect may

also be due to the lower toxicity of the aminoguanidine-formaldehyde complex.

It has generally been assumed that the enzymes responsible for the oxidation of formaldehyde are the same which oxidize other aldehydes and the meagre information we have regarding them has mostly been obtained from the specificity studies of acetaldehyde-metabolizing enzymes. The enzymes of animal origin which are known to catalyse *in vitro* also the oxidation of formaldehyde are aldehyde dehydrogenase, xanthine oxidase, aldehyde oxidase, glyceraldehyde-3-phosphate dehydrogenase, catalase and formaldehyde dehydrogenase.

Battelli and Stern demonstrated (1910 c) that animal tissues catalyse the so-called Cannizzaro's reaction, or dismutation of two moles of aldehyde to one mole of alcohol and one mole of fatty acid. Parnas (1910) showed that the catalyst was a soluble enzyme which he named *aldehyde mutase*, and von Euler and Brunius found (1928) that cozymase was required for this reaction. Dixon and Lutwak-Mann (1937) partly purified the liver aldehyde mutase and obtained a preparation which was entirely free from xanthine oxidase, and had no demonstrable alcohol dehydrogenase activity. They concluded that their aldehyde mutase was only one enzyme with two centres of activity. Adler, von Euler and Günther (1938) investigated further the aldehyde mutase and found that it also is able to rapidly dismutate formaldehyde in the presence of diphosphopyridine nucleotide. It also had alcohol dehydrogenase activity when supplemented with flavoprotein. The isolation by Racker (1949) of diphosphopyridine nucleotide-dependent *aldehyde dehydrogenase* from liver has led, however, to the conclusion that the aldehyde mutase action very probably is due to the combined action of both alcohol dehydrogenase and aldehyde dehydrogenase and that there apparently exists no such enzymic entity as aldehyde mutase. The liver aldehyde dehydrogenase reacts also with formaldehyde, although only at one-half of the rate as with acetaldehyde (Racker 1949).

Kendal and Ramanathan (1951, 1952) studied the reactions of formaldehyde catalysed by the alcohol dehydrogenase preparation of Lutwak-Mann (1938), which contains also aldehyde mutase activity. They confirmed the earlier findings that formaldehyde can be dismuted in this system when fortified with diphosphopyridine nucleotide. When also methanol or ethanol was added, the disappearance of formaldehyde was greatly accelerated and a volatile ester was formed. It was at first suggested that the appearance of methyl formate instead of formic acid in the formaldehyde + methanol system might be due to the presence of an amount of semiacetal sufficient to compete effectively with the aldehyde hydrate as a substrate for aldehyde dehydrogenase. The reaction, however, was quite insensitive to iodoacetate and this made the participation of aldehyde dehydrogenase in this reaction doubtful. It was therefore suggested by the authors that alcohol dehydrogenase may be capable of catalysing the complete dismutation by catalysing also the oxidation of a semiacetal

$CH_2O \cdot CH_3OH$ formed between the aldehyde and alcohol. More direct evidence is nevertheless needed before the mechanism of this reaction may be regarded as settled.

Milk and most animal tissues contain an enzyme, ***xanthine oxidase***, also called the Schardinger enzyme, which oxidizes both purines and aldehydes, reacting very rapidly with formaldehyde (SCHARDINGER 1902, MORGAN, STEWART and HOPKINS 1922, MORGAN 1926). It is a flavoprotein and reacts directly with oxygen (CORRAN, DEWAN, GORDON and GREEN 1939), but can transfer electrons to cytochrome *c* also (HORECKER and HEPPEL 1949). It has been shown recently that xanthine oxidase is a metalloflavoprotein containing molybdene (MACKLER, MAHLER and GREEN 1954). The metal group is required for reactions with cytochrome *c*, but not for reactions with oxygen or methylene blue.

Another flavoprotein enzyme acting on aldehydes was found in liver by GORDON, GREEN and SUBRAHMANYAN (1940) and called by them ***aldehyde oxidase***. It is very similar to xanthine oxidase but has no activity towards purines. It is also a molybdoflavoprotein requiring molybdene in its reactions with cytochrome *c* (MAHLER, MACKLER, GREEN and BOCK 1954). It is not quite clear whether this enzyme can use formaldehyde as substrate, as the enzyme is rapidly denatured in the presence of formaldehyde (GORDON *et al.* 1940). However, some activity is obtained when formaldehyde is used as substrate (CARPENTER 1951).

Glyceraldehyde-3-phosphate dehydrogenase, also called triose phosphate dehydrogenase, is an enzyme which catalyses the oxidative phosphorylation of D-glyceraldehyde-3-phosphate to 1 : 3-diphosphoglyceric acid (WARBURG and CHRISTIAN 1939, CORI, SLEIN and CORI 1948). It has been found to catalyse also the oxidative phosphorylation of acetaldehyde, propionaldehyde and butyraldehyde (HARTING 1951, HARTING and VELICK 1954). Oxidation of aldehydes can also occur in the absence of phosphate, although more slowly, and corresponding acids rather than phosphoric acid derivatives are formed. NYGAARD and SUMNER (1952) have reported that also formaldehyde is oxidized by glyceraldehyde-3-phosphate dehydrogenase in the presence of diphosphopyridine nucleotide, but the reaction products were not identified. During the last few years the mechanism of glyceraldehyde-3-phosphate dehydrogenase action has been a subject of active investigation, but no studies have been reported where formaldehyde was used as substrate. The affinity of the enzyme for acetaldehyde has been found to be very low and the physiological significance of this enzyme in the oxidation of non-phosphorylated aldehydes is still uncertain.

It has also been suggested that *catalase* takes part in the oxidation of formaldehyde *in vivo* (CHANCE 1951) since the catalase-hydrogen peroxide complex reacts *in vitro* very rapidly with methylene glycol (formaldehyde) (CHANCE 1948).

STRITTMATTER and BALL have recently (1955) identified a specific diphosphopyridine nucleotide-dependent ***formaldehyde dehydrogenase*** in

chicken and beef liver preparations. The enzyme is distinct from acetaldehyde dehydrogenase. Glutathione seems to be a specific cofactor in this system and it is suggested that formaldehyde reacts first with reduced glutathione to form *S*-hydroxymethylglutathione, which is then attached to the enzyme and oxidized. The product can be released from the enzyme either directly as formate, or as a free thiolester, which is subsequently hydrolysed to formate and glutathione. This scheme, however, is for the present hypothetical only and further studies have not yet been reported.

Condensation Reactions of Formaldehyde

As an aldehyde, formaldehyde can readily undergo condensation reactions with other substances. These condensation reactions have only lately gained interest also in the field of intermediary metabolism.

The addition of pyruvate or other α-ketoacids has been found to enhance greatly the rate of disappearance of formaldehyde in rat liver homogenates (P. Mitchell and Artom 1951). The reaction was interpreted as a condensation between formaldehyde and pyruvate. When the large granule fraction of rat liver was used, little or no formaldehyde disappeared unless pyruvate or other α-ketoacids were added (M. C. Mitchell and Artom 1952). The enzyme had an optimum pH of around 8 and was quite stable. No activitation was obtained by adding Mg^{++}, thiamine or cocarboxylase and no CO_2 was evolved during the reaction, which therefore differs from the formation of acyloins from pyruvate and higher aldehydes by tissue carboxylases. This enzyme is probably identical with the *formaldehyde-pyruvic acid carboligase* of Hift and Mahler (1952, Hift 1952), which catalyses an aldol condensation between formaldehyde and pyruvic acid. The product was identified as α-keto-γ-hydroxybutyric acid. This enzyme was present in whole tissue homogenates of rat kidney and liver but not of heart, diaphragm, spleen or brain. It was concentrated in the mitochondrial fraction, and required no external activating mechanism. Neither formaldehyde nor pyruvate disappeared when incubated singly with the enzyme. Phenylpyruvic acid reacted also with the enzyme but α-ketoglutaric acid did not.

Formaldehyde is also able to condense enzymically in the animal organism with substances other than pyruvic acid. According to Meyerhof, Lohman and Schuster (1936), aldolase can catalyse the condensation of formaldehyde with dihydroxyacetone phosphate during its formation from hexose diphosphate, but the reaction was not studied in detail. Charalampous and Müller (1953) studied this reaction with the aid of isotopic formaldehyde and found that a soluble enzyme system from rat liver catalyses the formation of erythrulose phosphate by the condensation of formaldehyde with a triose phosphate. This enzyme, which has been called *phosphoketotetrose aldolase* and which apparently differs from the Meyerhof-Lohman aldolase, has later been purified 40-fold, is not sensitive to

iodoacetic acid, and requires no added cofactors (CHARALAMPOUS 1954). The enzyme acts only with dihydroxyacetone phosphate and not with glyceraldehyde phosphate. In rat liver, kidney and muscle there is also found a soluble enzyme system which catalyses the anaerobic utilization of formaldehyde in the presence of erythrulose-1-phosphate (CHARALAMPOUS 1955). Ribose phosphate was identified as the major product of this reaction.

When homocysteine is added to sarcosine incubated with mitochondria the accumulation of metabolic formaldehyde is reduced and a new compound is formed (WRISTON 1955). It was concluded that this compound is the six-membered ring, *m*-thiazane-4-carboxylic acid. HARRIS and MACKENZIE (1955) reported that in their washed mitochondrial system formaldehyde and cysteine condense to form thiazolidine carboxylic acid, which is then dehydrogenated by a specific oxidase to yield the corresponding thiazoline, which in turn is hydrolysed to *N*-formylcysteine. Thus in the course of its oxidation the formaldehyde carbon undergoes a transfer from the sulphur to the amino group of cysteine.

Except with sulphydryl and keto groups, formaldehyde can also under certain conditions combine with the guanidino and amide groups and with the indolyl ring. It is also known that formaldehyde reacts rapidly at neutrality or in alkaline solutions with amino groups, forming aminomethylol groups (FRENCH and EDSALL 1945, FRAENKEL-CONRAT and OLCOTT 1946). These various condensation reactions of formaldehyde may partly aid in its metabolism but especially in the *in vitro* experiments they may also seriously reduce the effective concentration of reactive formaldehyde.

SIGNIFICANCE OF FORMALDEHYDE IN THE METABOLISM OF ONE-CARBON COMPOUNDS

Formaldehyde occupies, with formic acid, a central place in the metabolism of the so called one-carbon group. The carbon atom of formaldehyde can be incorporated into the methyl groups of choline in the intact rat and so be a precursor of labile methyl groups (DU VIGNEAUD, VERLY and WILSON 1950, JONSSON and MOSHER 1950, DU VIGNEAUD, VERLY, WILSON, RACHELE, RESSLER and KINNEY 1951). Formaldehyde can also be used in rat liver homogenates for the synthesis of the methyl group of methionine (SIEGEL and LAFAYE 1950). Also the ureido carbon atoms 2 and 8 of the purine ring can be derived from formaldehyde (MARSH 1951, GREENBERG 1951, 1953). Various liver preparations utilize formaldehyde for the synthesis of the β-carbon atom of serine, in which reaction tetrahydrofolic acid functions as cofactor (SIEGEL and LAFAYE 1950, KISLIUK and SAKAMI 1954, 1955, BLAKLEY 1954 a, b, ALEXANDER and GREENBERG 1955).

Although the reactions of formaldehyde and of formic acid are very similar, it has been found that they are not interconvertible in all of their reactions and that it is not necessary for formaldehyde to be first converted

to formate. Formic acid and formaldehyde are not necessarily interconverted in their incorporation into methionine methyl groups (BERG 1953) or into purine carbons 2 and 8 (GREENBERG 1951, 1953) but are possibly metabolized via a common intermediate. Formaldehyde but not formic acid can be added to glycine to form serine in pigeon liver preparations (BLAKLEY 1954 a, b). Experiments with serine doubly labelled in the β-position with radioactive carbon and deuterium indicated that in the synthesis of labile methyl groups from the β-carbon atom of serine both hydrogen atoms were transferred bound to the carbon atom (ELWYN, WEISSBACH and SPRINSON 1951, ELWYN, WEISSBACH, HENRY and SPRINSON 1955). This finding was not compatible with the existence in these reactions of an intermediate at the oxidation level of formate, as for example the citrovorum factor (5-formyl- 5: 6: 7: 8-tetrahydropteroylglutamic acid) which was generally implicated in the one-carbon group reactions as a carrier of the formyl group. In view of these results WELCH and NICHOL (1952) proposed that in addition to a formyl derivative of folic acid, which functions in the transfer of an intermediate at the oxidation level of formate, there is also a similar hydroxymethyl derivative of tetrahydrofolic acid which can be formed from an intermediate at the oxidation level of formaldehyde. There is also other evidence for the existence of these two folic acid derivatives (see e.g., BLAKLEY 1954 b, KISLIUK and SAKAMI 1955). BERG (1953) has proposed that homocysteine might function as the carrier of formyl and hydroxymethyl groups.

KISLIUK and SAKAMI (1955) have presented these complex interrelationships between the one-carbon compounds in a scheme which is repeated here in slightly modified form in figure 1.

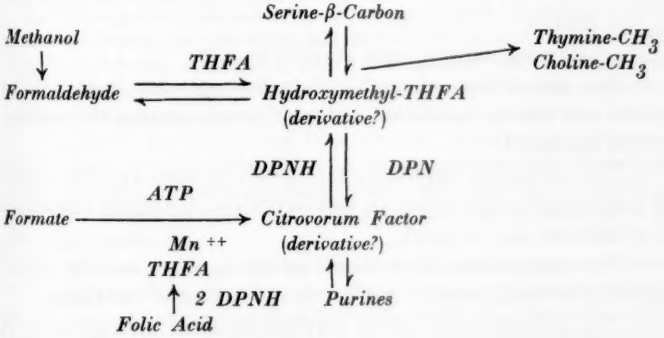

Fig. 1. — Interrelationships between one-carbon compounds. (Slightly modified from KISLIUK *and* SAKAMI *1955.) THFA = tetrahydrofolic acid.*

PRESENT INVESTIGATION

SUBJECTS OF INVESTIGATION

From the survey of the literature it was seen that although there are numerous investigations which in some way are connected with the metabolism of methanol, our knowledge of the actual metabolism of methanol in the animal organism is rather meagre. Especially the quantitative aspects of the metabolism of methanol in *in vitro* systems are very little known.

It was the purpose of this study originally to investigate the metabolism of methanol and formaldehyde in the animal organism at three different levels. First with *in vivo* techniques in intact animals, secondly with *in vitro* techniques in whole tissue preparations, and thirdly *in vitro* with purified enzyme preparations. Recent advances in this field, however, made modifications in this plan necessary and the present report therefore concerns only the *in vitro* studies with whole tissue preparations and the *in vivo* studies.

The following subjects were studied *in vitro*:

— The metabolism of methanol in homogenates from animal tissues and the accumulation of formaldehyde during the utilization of methanol;

— The optimum conditions for these reactions and the effect of various metabolic cofactors and inhibitors, especially the effect of disulfiram and ethanol;

— The metabolism of methanol in tissue slices and the accumulation of formaldehyde during the utilization of methanol;

— The utilization of added formaldehyde in homogenates of animal tissues.

The following subjects were studied in rabbits *in vivo*:

– The elimination of perorally or intravenously administered methanol from the blood and the possible accumulation of formaldehyde in the blood of these animals;

– The influence of ethanol and disulfiram on the elimination of methanol from the blood and on the possible accumulation of formaldehyde in the blood;

– The excretion of methanol and formic acid in the urine of methanol-treated rabbits and the effect of ethanol and disulfiram on this excretion.

EXPERIMENTAL TECHNIQUES

In vitro Experiments

Preparations from guinea pig and rat tissues were used in the *in vitro* experiments. The rats were of Wistar strain and the guinea pigs were of a strain inbred for a long time in this laboratory.

In most experiments the animals were fasted for one night, killed with a blow on the head without anaesthesia and bled. The organs were rapidly removed and cooled.

The tissues were used in the form of homogenates and slices. The word homogenate is used here in the conventional biochemical sense and does not indicate real homogenicity. For preparation of the homogenates the tissues were cooled on crushed ice or frozen with the aid of solid carbon dioxide immediately after killing of the animal. They were weighed and homogenized, using cold 0.1 M potassium phosphate buffer pH 7.4 as the homogenization medium.

The homogenizing device used in the preliminary experiments was a mixer of Waring blendor type, but better and more consistent results were attained with a refrigated Bühler homogenizer, which therefore was used in the greater part of this study. The homogenizer was cooled with ice water, the speed 10 was used and the homogenization time was 30 seconds except for skeletal and heart muscles, which were homogenized for 45 seconds. After homogenization the preparation was diluted with buffer to the required concentration, which in most experiments was 10 per cent (wet weight of tissue by volume). It was used immediately if not otherwise stated.

The slices were prepared from tissues cooled on crushed ice and kept moist with physiological saline. They were cut freely by hand with a cut-throat razor and only the most uniform slices were used. They were rapidly drained and weighed on a torsion balance and introduced into the reaction vessels with the smallest possible delay.

The following buffers were used:

0.1 M potassium phosphate buffer pH 7.4 was prepared by mixing appropriate amounts of 0.1 M primary and secondary potassium phosphate solutions. This buffer was used in most experiments.

0.2 M phosphate buffer according to Sörensen was prepared from 0.2 M secondary sodium phosphate and 0.2 M primary potassium phosphate solutions.

0.2 M pyrophosphate buffer was prepared from 0.2 M sodium pyrophosphate and 0.2 M hydrochloric acid.

The pH values of potassium phosphate and pyrophosphate buffers were checked repeatedly with a »Radiometer» pH meter, using as primary standard a buffer mixture of pH 6.81 prepared of equal volumes of 0.1 M secondary sodium phosphate and 0.1 M primary potassium phosphate.

The substrates, activators and inhibitors were as follows:

Methanol. — Aqueous solutions were prepared from a *pro analysi* product of Merck. Since no difference was noted in the experiments whether or not the methanol was redistilled, it was used directly. Specific gravity 0.795 was assumed.

Formaldehyde. — Formaldehyde (Merck, p.a.) was distilled and the distillate was analysed for the formaldehyde content and diluted with water to the concentration required in each experiment.

Diphosphopyridine nucleotide. — This was a commercial preparation manufactured by Boehringer und Söhne. In the preliminary experiments, diphosphopyridine nucleotide prepared from yeast according to Sumner, Krishnan and Sisler (1947) was used.

Adenosine triphosphate. — In most experiments Light Co.'s preparation and in some experiments that of Gedeon Richter (Budapest) was used.

Diphosphothiamine. — This was Berolase (LaRoche-Hoffmann).

Glutathione. — Reduced glutathione from Schwarz Co. was used.

Cytochrome c. — A preparation manufactured by Light Co. was used.

Disulfiram. — Crystalline tetraethylthiuram disulphide was used.

All the other reagents used were *pro analysi* products of Merck or of a corresponding quality.

The incubations were carried out in 50 ml Erlenmeyer flasks at + 37° in a temperature-controlled water bath. The flasks were mechanically shaken during the incubation.

The reaction mixture was usually composed of 10 ml of homogenate (or 10 ml of buffer and 0.7 — 2.0 g of slices, wet weight) and 2 ml of substrate and other additions. Buffer was added until the total volume was the same in all the flasks in each series, usually 12 ml or 11 ml. The ingredients were added to the flasks in the following order: the additions (inhibitors, activators, etc.), the tissue preparation, and the substrate. During the preparation of the reaction mixture the flasks were immersed in ice water.

When oxygen or nitrogen was used as the gas phase the flasks were gassed for 5 minutes after the reaction mixtures were ready. No loss of substrate during gassing was observed when formaldehyde was used as

substrate. When methanol was used a loss of 5 to 10 per cent was observed and was corrected with the aid of one or two control flasks in each series, which were analysed immediately after gassing. Ordinary tank oxygen was used. The nitrogen was tank nitrogen passed through FIESER's (1924) solution to free it from possible traces of oxygen.

Samples were taken before and after the incubation. In some experiments a number of samples were taken also during the incubation. The samples, usually 1 ml, were transferred to centrifuge tubes containing 1 ml of 20 per cent trichloroacetic acid for the precipitation of proteins. After standing for about one-half hour 1 to 2 ml of water were added and the tubes cooled and centrifuged. The clear supernatant obtained was used for the analyses, which were performed in duplicate.

In vivo EXPERIMENTS

The experimental animals were white and brown rabbits about 2—3 years old and weighing 2.5 to 3.5 kg. The diet during the experiments was oats and water *ad libitum*.

The peroral administration of alcohols and disulfiram was made through a rubber stomach tube. The alcohols were diluted with water to a 15—20 per cent solution and the disulfiram was given as a 15 per cent aqueous suspension stabilized with a drop of Tween 80.

For intravenous administration into the ear vein the alcohols were diluted with physiological saline *pro injectione* (Orion) to make 10 ml, which was given as a slow injection during 10—15 minutes. More rapid injection, especially of the higher concentrations, tended to kill the animals.

The blood samples, usually 0.6 ml, were taken from the ear vein with a recalibrated 1 ml tuberculin syringe containing 0.4 ml heparin solution (125 μg heparin per ml). After thorough mixing the sample was transferred to a centrifuge tube containing 1 ml of 20 per cent trichloroacetic acid. After standing for one-half hour 2 ml of water were added and the tubes cooled and centrifuged. The clear supernatant obtained was used for the analyses, which were performed in duplicate.

ANALYTICAL METHODS

DETERMINATION OF FORMALDEHYDE

A wide variety of methods are available for the determination of the highly reactive formaldehyde. However, many of these methods are not specific for formaldehyde, being based on general aldehyde reactions or being still more unspecific. The methods suitable for biochemical analysis, where many disturbing factors are present, must have a rather high specific-

ity and they must also be sufficiently sensitive for the determination of very small formaldehyde concentrations.

The discovery of DENIGÈS (1896, 1910 a) that Schiff's fuchsin-bisulphite reagent, a general reagent for the detection of aldehydes, gives a specific colour with formaldehyde when employed in the presence of strong acids, has led to several more or less modified methods for the determination of formaldehyde. These methods have the advantage of being both extremely simple and fairly specific. The sensitivity of these photometric procedures is about 1 : 1 000 000.

SCHRYVER reported in 1910 a method for estimating formaldehyde based on a reaction with phenylhydrazine hydrochloride. The product, when oxidized with potassium ferricyanide and acidified with hydrochloric acid, gives a red colour. DOWSE and SAUNDERS (1955) have recently studied this reaction and determined the optimum conditions for its use in the quantitative determination of formaldehyde.

TANENBAUM and BRICKER (1952) have described a method for the micro-estimation of free formaldehyde, which is based on the formation of a red colour by the reaction of formaldehyde with phenylhydrazine and potassium ferricyanide. However, acetaldehyde interferes with this reaction, as also do methanol and ethanol, which are partly oxidized to aldehydes by the employed ferricyanide.

NASH (1953) has described a method for the colorimetric estimation of formaldehyde by means of the Hantzsch reaction comprising the synthesis of dimethyldihydrolutidine from acetylacetone and formaldehyde in the presence of an excess of ammonium salt.

Formaldehyde can also be estimated gravimetrically as its dimedone derivative (VORLÄNDER 1929, WEINBERGER 1931). However, the sensitivity of this method is not very high; furthermore, other aldehydes will also give a dimedone derivative and the quantitative separation of these non-specific materials without loss of the formaldehyde derivative seems to be difficult.

EEGRIWE observed (1937) that formaldehyde reacted with chromotropic acid (1 : 8-dihydroxynaphthalene-3 : 6-disulphonic acid) on being heated in strong sulphuric acid, yielding a highly violet coloured compound, and that the reaction appeared to be specific for formaldehyde. This reaction has since been adapted for quantitative use by several workers (BOYD and LOGAN 1942, MACFAYDEN 1945, BRICKER and JOHNSON 1945, and others).

In the present study the chromotropic acid method was used for the determination of formaldehyde. Some preliminary experiments were also made by the methods of TANENBAUM and BRICKER (1952) and that of NASH (1953) but they seemed to offer no advantages over the chromotropic acid method, which was found to be superior in many respects. The results obtained with the Hantzsch reaction were not uniform. The phenylhydrazine reaction is very time-sensitive and therefore was not well suited for serial determinations. The interference of methanol and ethanol also made it unsuitable for these studies.

The procedure adopted in the present work for the determination of formaldehyde is described below.

Reagents:

0.20 per cent chromotropic acid solution (in 11.5 M-H_2SO_4). — Chromotropic acid (Merck) was used. The solution was stable for at least one week if stored in a brown bottle in the refrigerator. During this time the blank values increased somewhat but this did not disturb the analysis. In most determinations, however, a freshly made solution was used.

11.5 M-H_2SO_4. — This was prepared from concentrated H_2SO_4 (p.a., Merck).

Formaldehyde stock standard solution. — This was prepared by hydrolysing 778.5 mg of hexamethylenetetramine (Merck) in 50 ml of 10 N-H_2SO_4 for 2 days at room temperature, followed by dilution to 500 ml with distilled water. Thus 1 ml of this stock standard corresponds to 2 000 μg formaldehyde. The stock standard solution was very stable when stored in the refrigerator. Working standards were prepared from this stock standard when needed, by diluting with distilled water. A dilution series containing 1, 2, 3, and 4 μg formaldehyde per ml was mostly used for the standardization of the determination.

Procedure:

Into a glass stoppered test tube was pipetted 0.5 ml of the protein-free sample to be analysed. If more than 10 μg formaldehyde per ml was expected the sample was first appropriately diluted with distilled water. Then 3 ml of 0.20 per cent chromotropic acid solution in 11.5 M sulphuric acid were added and the tubes were immediately closed and shaken. The tubes were heated in a water bath at 80° for 1 hour. After cooling the optical densities were determined with a Beckman B spectrophotometer at 570 mμ.

The values for formaldehyde content were calculated using a coefficient obtained from several series of standard determinations. Standard determinations were made about once a week and always after adopting new batches of reagents. The coefficient (optical density of 1 μg formaldehyde minus that of the reagent blank) was 0.151 ± 0.0068, as calculated from all the 104 standard determination series made. The variations within a single series of standards were negligible and a linear correlation was always obtained between the optical density and the formaldehyde concentration.

The analyses were performed directly from suitably diluted protein-free samples. In addition to the usual reagent blank, a tissue blank was always used in the determinations from homogenates and slices. This was made from tissue preparation which was incubated without substrate but was otherwise treated like the actual experiments.

When known amounts of formaldehyde were added to deproteinized

homogenates the recovery of formaldehyde was found to be quantitative. If formaldehyde was added to homogenates before deproteinization a part of the formaldehyde was bound to the homogenate, the amount being correlated to the concentration of the homogenate. When 10 per cent liver homogenates were used, about 15—25 per cent of the added formaldehyde was bound to it and the recovery was thus only 75—85 per cent.

In the actual experiments on the utilization of formaldehyde this effect was eliminated by taking samples also before incubation to ascertain the initial composition of the reaction mixture and by using boiled homogenates as controls for the non-enzymic disappearance of formaldehyde. In the experiments in which the accumulation of formaldehyde from methanol was followed no such elimination of the effect of the non-enzymic binding of formaldehyde was possible. The values for formaldehyde are not corrected for this effect in any of the experiments.

Determination of Methanol

The most suitable methods for the determination of methanol in biochemical work are based on its oxidation. Either the used oxidant is determined or the formaldehyde, which under proper conditions is the principal oxidation product, is determined by some of the methods dealt with above.

Widmark's (1922) micromethod for ethanol determination was modified by Bildsten (1924) for the determination of methanol. He determined the constant k which gives the correlation between used bichromate and the amount of methanol present. However, the value for k which he gives, namely 1. 33 for 1/200 N thiosulphate, is apparently about 5 times too high although he obtained with this method very plausible results in methanol determinations from rabbit blood. Neymark (1936) and Schmidt (1949) give as the correct value 0.56 for 1/100 N thiosulphate; Seifert and Günther (1951), however, use 0.7 as the value for k. If ethanol or other reducing volatile agents are present this method is not applicable, and also if both methanol and formaldehyde are present together this method cannot be used.

The procedures based on the oxidation of methanol to formaldehyde, followed by photometric or colorimetric determination of formaldehyde are the most widely used methods. The oxidating agent is usually acid potassium permanganate and the oxidation is stopped with oxalic acid or bisulphite. The formaldehyde is usually determined with modified Schiff's reagents, and several more or less modified variations of the original method of Denigès (1910 b) are used for the determination of methanol (e.g., Wright 1927, Ant-Wuorinen 1935). Ethanol is usually added to sensitize the reaction. The procedure is sensitive to temperature variations and should be carried out in a thermostat. Another possibility is to use chromotropic acid for the determination of formed formaldehyde.

This method has been adapted for blood methanol determination by AGNER and BELFRAGE (1947), who used acid permanganate as oxidant and stopped the oxidation with sulphur dioxide. Also BOOS (1948) and FELDSTEIN and KLENDSHOJ (1954) have proposed similar methods, and the latter authors have combined it with microdiffusion in a Conway unit.

The chromotropic acid method seemed most suitable for use in the present study because of its simplicity and sensitivity. The optimum conditions for the chromotropic acid reaction were first studied. The effects of such factors as the concentration of sulphuric acid and chromotropic acid and the incubation temperature and time were studied. The results obtained by MACFAYDEN (1945) and BRICKER and JOHNSON (1946) were confirmed. The optimum conditions for the oxidation of methanol to formaldehyde were also investigated. The results obtained were similar to those of AGNER and BELFRAGE (1947).

In the procedure adopted for methanol determination the incubation time was increased to 60 minutes, since in some direct determinations from tissue samples the maximum colour was not developed until after 30 minutes. The incubation temperature was decreased to 80° in order to diminish the brownish discolourations readily developed from the more concentrated tissue samples when incubated with sulphuric acid at 100°. The total volume was 3.7 ml, which just fits the cuvettes for the Beckman photometer. A 10 per cent solution of sodium bisulphite was used for reducing the excess permanganate. Sodium arsenite was also tried as a reducing agent but it reacted with permanganate, giving an intense red colour and therefore could not be used.

The procedure used for the determination of methanol is described below.

Reagents:

In addition to the reagents for the determination of formaldehyde (p. 33) the following were used.

Potassium permanganate solution. — 6 ml of sirupous orthophosphoric acid *pro analysi* (UCB) were diluted with distilled water to 100 ml. This solution was saturated with potassium permanganate (p.a., UCB).

Bisulphite solution. — A 10 per cent solution of sodium metabisulphite (p.a., Merck) was used. This solution must be freshly made.

Standard methanol solutions. — Methanol (p.a., Merck) was used. The standard dilutions used contained 20, 30, 40 and 50 μg methanol per ml. The specific gravity of methanol was assumed to be 0.795.

Procedure:

Of the protein-free sample to be analysed, which may contain up to 120 μg methanol per ml, 0.5 ml is pipetted into a glass stoppered test tube. Then 0.1 ml of the acid potassium permanganate solution is added, the

tube closed and the oxidation left to proceed for 20 minutes at room temperature. Care must be taken that all of the sample is in contact with the oxidizing solution. After 20 minutes 0.1 ml of bisulphite solution is added to stop the oxidation and decolourize the permanganate. Thereafter 3 ml of 0.20 per cent chromotropic acid solution in 11.5 M sulphuric acid are added and the tubes are closed and well shaken. After 1 hour in a water-bath at 80° the tubes are allowed to cool and then the optical densities are read at 570 mμ with the aid of a Beckman B spectrophotometer.

The methanol values were calculated with the aid of a coefficient obtained from determinations of known methanol concentrations. The optical density of 1 μg methanol minus that of the reagent blank was 0.0134 ± 0.0012, as calculated from the 86 standard series made. Standard determinations were run about once a week, and always after adopting new batches of reagents. A linear correlation was always obtained between the optical density and the methanol concentration.

All the methanol determinations were carried out directly from the suitably diluted protein-free samples, with the exception of those for urine, in which the distillation procedure of LEAF and ZATMAN (1952) was used for the isolation of methanol. A tissue blank, incubated without substrate, was always used in the determinations from tissue preparations. The recovery of added methanol from deproteinized 10 per cent liver homogenates was 97—105 per cent and no correction for it was made in the actual experiments. When known amounts of methanol were added to non-deproteinized homogenates a part of the methanol was bound to the homogenate. The amount of bound methanol was correlated to the concentration of the homogenate and was about 20 per cent when 30 per cent homogenate was used. When 10 per cent homogenates were used the recovery was almost quantitative. Since samples of the reaction mixture were always analysed also before incubation, and since the experiments with boiled homogenates demonstrated that no more methanol was bound during the incubation, the effect of the fixation of methanol in the tissue preparations on the final results was eliminated.

Analysis of Mixtures of Methanol and Formaldehyde. — When the sample to be analysed contains both methanol and formaldehyde, the usual manner of analysis does not give the correct values. Methanol does not react in the formaldehyde determination, and the values obtained for the formaldehyde content in mixtures of formaldehyde and methanol are therefore valid. Formaldehyde, however, reacts also in the methanol determination, and excessively high values are obtained. It has been proposed that in such cases it is possible to use the difference between an oxidized and a non-oxidized sample as the value for methanol (FELDSTEIN and KLENDSHOJ 1954). However, neither will this give the correct values since acid permanganate also oxidizes a part of the formaldehyde present, and, on the other hand, the oxidation of methanol is not quantitative. The oxidation

of formaldehyde by acid permanganate was therefore studied in order to be able to calculate the formaldehyde corrections in the methanol values for mixtures of methanol and formaldehyde.

When known amounts of formaldehyde were carried through the methanol determination procedure it could be established that 1 μg of formaldehyde in the methanol determination is equal to 2.5 μg of methanol. Therefore, when also formaldehyde was present, 2.5 times its concentration was subtracted from the value obtained for methanol. In table 1 are shown

TABLE 1

Analysis of known mixtures of methanol and formaldehyde

Methanol			Formaldehyde		
Theory μg	Found μg	Recovery per cent	Theory μg	Found μg	Recovery per cent
10	9.6	96	0	0.00	—
10	10.1	101	0	0.00	—
10	10.1	101	0.50	0.41	82
10	10.3	103	0.50	0.48	96
10	10.0	100	1.00	1.02	102
10	9.6	96	1.00	0.97	97
10	9.9	99	1.50	1.47	98
10	10.5	105	1.50	1.38	92
10	10.5	105	2.00	2.02	101
10	9.5	95	2.00	1.93	97
5	4.2	84	1.00	0.93	93
5	4.0	80	1.00	0.95	95
5	5.9	118	2.00	1.85	93
5	4.3	86	2.00	1.94	97
5	4.7	94	2.50	2.44	98
5	4.4	88	2.50	2.50	100
0	0.0	—	1.00	0.96	96
0	0.0	—	1.00	1.02	102

the results obtained with known mixtures of formaldehyde and methanol analysed in the above manner. It will be observed that good recovery was obtained.

Interfering Substances. — Ethanol, disulfiram, adenosine triphosphoric acid, diphosphopyridine nucleotide, glutathione, diphosphothiamine, monoiodoacetate, malonate, pteroylglutamic acid and vitamin B_{12} did not interfere with the formaldehyde and methanol determinations in the concentrations used. In table 2 are presented the results of experiments in which the possible interference of certain other substances with the determination of formaldehyde and methanol was investigated. It is seen that the concentrations possibly present in tissue preparations have no significant effect on these determinations. However, if fructose, glucose or ascorbic acid are added into the reaction mixture they may invalidate

TABLE 2

Effect of various compounds on the determination of methanol and formaldehyde
Pure solutions were used for the determinations.

Compound	Concentration mg per ml	Methanol µg per ml	Formaldehyde µg per ml
Li-lactate	0.50	0	0.0
»	2.50	7	0.0
Na-pyruvate	0.25	1	0.0
»	0.50	1	0.0
»	2.50	3	0.0
»	5.00	11	0.0
Glucose	0.50	1	0.1
»	2.50	11	0.5
»	5.00	16	1.0
Ascorbic acid	0.025	0	0.0
»	0.125	1	0.2
»	0.500	2	0.5
»	0.750	2	0.8
»	1.000	3	1.1
Fructose	0.005	0	0.0
»	0.125	5	0.7
»	0.250	11	1.2
»	0.500	20	2.3

the results of direct analyses of formaldehyde and methanol. As sugars, they react with sulphuric acid, and fructose is also oxidized to formaldehyde by acid permanganate.

DETERMINATION OF FORMIC ACID

Most methods which have been used for the determination of formic acid are based on the fairly specific oxidation reaction with mercuric chloride. When heated with mercuric chloride, formic acid is oxidized to carbon dioxide and water and the mercuric chloride is thereby reduced to mercurous chloride. The mercurous chloride formed can then be determined gravimetrically (POHL 1893, FINCKE 1913) or iodometrically (RIESSER 1916, 1923). Volumetric determination of the formed carbon dioxide was used by PIRIE (1946). GRANT (1947) determined the mercurous chloride colorimetrically by measuring the colour formed when the mercurous chloride was treated with a phosphotungstic-phosphomolybdic acid reagent. The sensitivity was thus increased about 100-fold.

DROLLER (1932) determined formic acid colorimetrically after reduction to formaldehyde with fuchsin-sulphurous acid. He used as reducing agent

hydrogen generated by magnesium in hydrochloric acid. Chromotropic acid has also been used for the determination of formaldehyde formed in the reduction of formic acid (GRANT 1948).

In the methods for the determination of formic acid in urine, blood or other biological substances it is necessary first to isolate the formic acid. In most methods either steam distillation (POHL 1893, FINCKE 1913, PIRIE 1946) or vacuum distillation (STRISOWER 1913, GRANT 1948) has been used. RIESSER (1916) combined ether extraction and steam distillation.

BASTRUP (1947) worked out a very practical method for the determination of formic acid in urine. The formic acid is isolated as a very volatile methyl formate after esterification with methanol using hydrogen ions as catalyst. The methyl formate is distilled into a sodium hydroxide solution, in which it is saponified, with reformation of formic acid. The actual determination of formic acid is then carried out by the procedure of RIESSER (1916, 1923), in which the mercurous chloride formed in the reaction of formic acid with mercuric chloride is determined iodometrically.

In the present study the method of BASTRUP (1947) was used for the determination of formic acid. The colorimetric methods of GRANT (1947, 1948) were used in preliminary experiments, but they did not give as consistent results as the method of BASTRUP.

RESULTS

METABOLISM OF METHANOL AND FORMALDEHYDE *IN VITRO*

The *in vitro* experiments which are presented in this section were mostly made with liver tissue homogenates, because it was found in preliminary experiments that methanol is readily utilized in these preparations and that formaldehyde accumulates during this reaction. The homogenate technique was also technically more convenient and made possible a more rigid control of outer conditions than the tissue slice technique, which, however, was used in some experiments. For comparison, other tissues than liver were also studied. Due to external circumstances both rat and guinea pig tissues had to be used interchangeably in some experiments. No qualitative differences between them were noted, however, and the results may be regarded as comparable qualitatively in spite of the different animal species used.

In general the variations in the activity of tissue preparations obtained from different animals and at different times were considerable. This variation, however, was very small within a single experimental series, in which the same tissue preparation was used under identical conditions in every experiment. Therefore in interpreting the results, only the experiments in the same series can be directly compared to each other in a quantitative sense. Despite the above mentioned variation, the results show the same qualitative and quantitative tendencies also when different series are compared.

The values presented for the utilization of methanol and accumulation of formaldehyde refer to the difference between the values found before and after the incubation. All the values are

calculated per initial volume. Usually about 3—6 different series were made in each group and the results of one or more typical series are presented.

Because of the variations in the activity of the different preparations and the relatively small material, the data have not been analysed statistically.

Utilization of Methanol and Accumulation of Formaldehyde in Liver tissue Homogenates

Since very little is known concerning the quantitative aspects of the metabolism of methanol and formaldehyde in animal tissues it was first necessary to investigate the optimum conditions for these reactions. Liver tissue homogenates were used in the following experiments. The effect of fundamental factors such as pH of the reaction mixture, aerobic and anaerobic conditions, age of the homogenate, incubation time, concentration of the homogenate, and initial concentration of substrate have been studied.

Effect of pH

One of the essential characteristics of enzymic reactions is their dependence on the pH of the reaction mixture. In order to elucidate the dependence of the amount of methanol utilized in tissue homogenates on the pH of the reaction mixture the utilization of methanol was determined in guinea pig liver homogenate for the buffer pH range of 6.5 to 9.8. The accumulation of formaldehyde was also followed. If the enzyme or enzymes responsible for the utilization of formaldehyde do not have the same pH optima as the enzymes responsible for the utilization of methanol, this should presumably become manifest as a difference in the shape of the curves for the effect of pH on the utilization of methanol and accumulation of formaldehyde.

The arrangement of these experiments was as follows. Guinea pig liver was homogenized with water to a 20 per cent homogenate. Then 5 ml of this homogenate and 5 ml of 0.2 M buffer, pH 6.5—9.8, were incubated in air at 37° for 90 minutes with 1 600 μg of methanol and the utilization of methanol and accumulation of

formaldehyde were determined. Buffers used were 0.2 M ordinary phosphate buffer according to Sörensen (from primary potassium and secondary sodium phosphate) for the pH range 6.5—8.0, and 0.2 M sodium pyrophosphate buffer for pH range 8.0—9.8.

The effect of pH on the utilization of methanol is seen from figure 2. The optimum pH was between about pH 7.3 and pH 8.3 and no sharp maximum was found. On this pH range about 60 per cent of the added 1 600 μg of methanol was utilized during the 90 minutes' incubation. There was no difference in the values for ordinary phosphate buffer or pyrophosphate buffer at pH 8.0.

In figure 3 is shown the dependence of the accumulation of formaldehyde on pH in the same experiments. The shape of the curve is quite similar to the methanol utilization curve if the effect of pyrophosphate buffer described below is taken into account. The maximum accumulation is seen between pH values

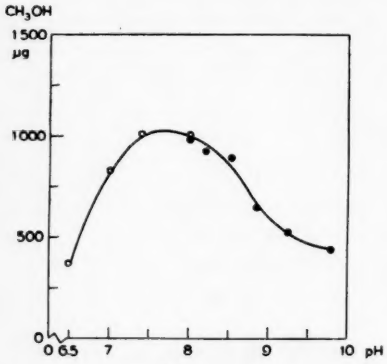

Fig. 2.

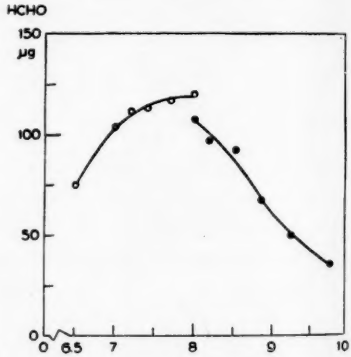

Fig. 3.

Fig. 2. — *Effect of pH on the utilization of methanol in guinea pig liver homogenate. Ordinate: Amount of methanol utilized. Abscissa: pH of buffer. 10 ml of 10 per cent homogenate in 0.1 M buffer.* ○ = *Sörensen's phosphate buffer;* ● = *pyrophosphate buffer. Total volume 11 ml. Substrate: 1 600 μg of methanol. Incubated for 90 minutes at 37°. Gas phase: air.*

Fig. 3. — *Effect of pH on the accumulation of formaldehyde in guinea pig liver homogenate incubated with methanol. Same experiments as in fig. 2. Ordinate: Amount of formaldehyde accumulated. Abscissa: pH of buffer.* ○ = *Sörensen's phosphate buffer;* ● = *pyrophosphate buffer.*

7.3 and 8.3. This similarity of the curves indicates the same optimum pH ranges for the enzymes responsible for the utilization of methanol and formaldehyde. Another explanation may be that the formation of formaldehyde from methanol is to such an extent a dominant factor in the accumulation of formaldehyde, as compared to the utilization of formaldehyde, that it alone determines the shape of the curve.

The accumulation of formaldehyde in pyrophosphate buffer was smaller than that in ordinary phosphate buffer. This effect was seen in all the experiments in which pyrophosphate and ordinary phosphate buffers were used under otherwise similar conditions. The difference was not very great, however, varying from 10 to 25 per cent. On the other hand, no significant effect was noted on the utilization of methanol. This result was very probably due to the increased utilization of formaldehyde in pyrophosphate buffer. MITOMA and GREENBERG (1952) also found in their isotope experiments that the formaldehyde formed in the oxidative demethylation of sarcosine in liver preparations was oxidized to carbon dioxide to a greater extent in pyrophosphate buffer than when other buffers were used.

Effect of Incubation Temperature

The incubations in all experiments were carried out in a temperature-controlled water bath at $+37°$.

In preliminary experiments it had been found that the utilization of methanol was dependent to a great extent on the incubation temperature. It was negligible when the homogenate was kept at 0°, and only very small amounts of methanol were utilized at room temperature. The accumulation of formaldehyde was also low at these temperatures, corresponding to the low utilization of methanol. Complete experimental series, however, were not made.

Effect of Aerobic and Anaerobic Conditions

To find out the extent to which the utilization of methanol and accumulation of formaldehyde are dependent on aerobic con-

ditions, experiments were made where the incubations were carried out under oxygen, atmospheric air and nitrogen.

The results of these experiments are recorded in table 3. It is

TABLE 3

Utilization of methanol and accumulation of formaldehyde in rat liver homogenates incubated in air, oxygen or nitrogen.

10 ml of 10 per cent homogenate in 0.1 M potassium phosphate buffer pH 7.4. Total volume 11 ml. Substrate 1 600 µg of methanol. Incubated 90 minutes at 37°. Different homogenates were used in experiments 1—6 and 7—10.

No.	Gas phase	Methanol utilized µg	Formaldehyde accumulated µg
1	Air	760	79
2	»	820	99
3	Oxygen	850	108
4	»	1040	105
5	Nitrogen	150	0
6	»	106	0
7	Air	785	116
8	»	920	126
9	Oxygen	804	131
10	»	754	129

seen that some methanol can be utilized by rat liver homogenates also when incubated under nitrogen, indicating that methanol can partly be utilized via anaerobic mechanisms. This effect may also be due to traces of oxygen left in the nitrogen used. The anaerobically utilized amount of methanol, however, is only about 15 per cent of the amount utilized under aerobic conditions. No formaldehyde accumulated when the incubation was carried out under nitrogen.

No difference was clearly seen in the utilization of methanol in air and in oxygen. The amount of oxygen in atmospheric air seemed to be adequate to permit full utilization of methanol. However, somewhat more formaldehyde accumulated under oxygen than under air.

Because there were no significant differences between the results in air and oxygen, atmospheric air was used as the gas phase in most of the experiments in this study.

Effect of Ageing of the Homogenate

The following experiments were made to obtain some knowledge of the stability of the enzyme systems responsible for the utilization of methanol and formaldehyde in liver homogenates. Immediately after killing of the animal, guinea pig liver tissue was homogenized as usually to make a 10 per cent homogenate. In this case the homogenate was stored in a refrigerator at + 2° and portions of it were used for experiments after various lapses of time. The incubations were carried out using air as the gas phase. From table 4 it is seen that much more formaldehyde accumulated when

TABLE 4

Effect of ageing of the homogenate on the utilization of methanol and accumulation of formaldehyde in homogenates of guinea pig liver tissue

Homogenate stored at + 2°. 10 ml of 10 per cent homogenate in 0.1 M potassium phosphate buffer pH 7.4. Total volume 11 ml. Substrate 1 600 µg of methanol. Incubated for 90 minutes at 37°. Gas phase: air.

No.	Homogenate ageing time min.	Methanol utilized µg	Formaldehyde accumulated µg
1	20	850	130
2	20	900	115
3	360	910	150
4	360	870	175
5	720	820	195
6	720	850	220

methanol was incubated with stored liver homogenate than when fresh homogenate was used. This effect can be due either to increased formation or to decreased utilization of formaldehyde or to both. Since there was no clear difference in the utilization of methanol in the fresh and aged liver homogenate, the decreased utilization of formaldehyde seems the most probable of these alternatives.

The tissue blank values for both formaldehyde and methanol were found to increase during storage of the homogenate. When the storage time was 12 hours these blank values were approximately doubled. However, these values were still so small that they were of no importance in the final results. The blanks were brownish rather than of the violet formaldehyde colour. To find

out if the increase in the tissue blank values was nevertheless due to the formation of formaldehyde during storage, absorption spectra were made from the tissue blanks used in the formaldehyde determinations for fresh and aged homogenates. In the spectra for aged homogenate no special increase was seen in the absorption maximum of the formaldehyde-chromotropic acid colour, which is around 570 mμ. The increase in blanks was most probably due to some substance or substances other than formaldehyde, which were formed by autolysis of the tissue during the storage and which reacted with chromotropic acid or sulphuric acid to give a brown discolouration.

The stability of the enzyme systems was studied also at lower temperatures in order to see whether liver tissue could be stored deep frozen for later experiments or whether this would influence the reactions of the tissue. Guinea pig liver was used in these experiments. After killing of the animal a portion of the liver was immediately homogenized and used for incubation with methanol as usually. The remaining tissue was rapidly frozen with solid carbon dioxide and stored at −18°. Homogenates were prepared from this tissue after 24 and 48 hours and incubated with methanol. The results are presented in table 5. Only a small

TABLE 5

Effect of the storage of the tissue at −18° on the utilization of methanol and accumulation of formaldehyde in homogenates of guinea pig liver tissue
Substrate 1 600 μg of methanol. Experimental conditions as in table 4.

No.	Tissue storage time hr.	Methanol utilized μg	Formaldehyde accumulated μg
1	12	1000	110
2	24	980	100
3	36	1050	115
4	48	1090	120

increase in the accumulation of formaldehyde after storage of the tissue at −18° was seen and no effect on the utilization of methanol. There was also no demonstrable increase in the tissue blank values when the liver was stored in the frozen state. Liver tissue which

had been stored at −18° for 24 hours was therefore used in some experiments. However, fresh tissue was used in most of the experiments.

Effect of Incubation Time

To investigate also the time relationships of the utilization of methanol and accumulation of formaldehyde these reactions were followed in several series of experiments by taking samples during the incubation at various time intervals. The gas phase was air in all the experiments.

Curves for the utilization of methanol in rat liver homogenates during an incubation period of 120 minutes are presented in figure 4. It is seen that methanol was very readily utilized in these preparations and that the utilization was rather rapid during the

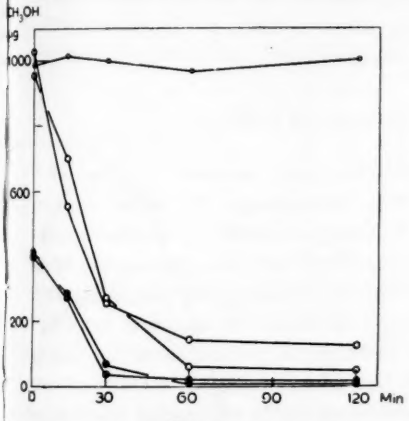

Fig. 4.

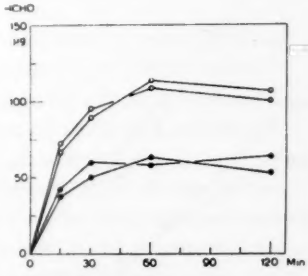

Fig. 5.

Fig. 4. — *Utilization of methanol in rat liver homogenate. Ordinate: Concentration of methanol. Abscissa: Incubation time. 10 ml of 10 per cent homogenate in 0.1 M potassium phosphate buffer, pH 7.4. Total volume 11 ml.* ○ — ○ *1 000 µg of methanol;* ○ — ○ *1 000 µg of methanol + boiled homogenate;* ● — ● *400 µg of methanol. Gas phase: air. Incubation temperature 37°.*
Fig. 5. — *Accumulation of formaldehyde in rat liver homogenate incubated with methanol. Same experiments as in fig. 4. Ordinate: Amount of formaldehyde accumulated. Abscissa: Incubation time. No formaldehyde was accumulated in the boiled homogenate incubated with 1 000 µg of methanol.*

first 30 minutes and then slowed down. During these 30 minutes about 75 per cent of the added 1 000 μg and 85 per cent of the added 400 μg were utilized. If the homogenate was first boiled no utilization of methanol was seen, which can be regarded as a demonstration of the enzymic nature of this reaction. In figure 6 are presented curves for the methanol utilization in another series of experiments where the reaction was followed for 210 minutes. The shape of the curves were similar in both experiments, although somewhat less methanol was utilized in the latter series.

The accumulation of formaldehyde in the same experiments is presented in figures 5 and 7. The direct relationship of the accumulation of formaldehyde to the utilization of methanol is clearly seen, the rate of formaldehyde accumulation being most rapid in the beginning of the experiments and then slowing down. From figure 7 it is seen that the formaldehyde concentration

Fig. 6.

Fig. 7.

F i g. 6. — *Utilization of methanol in rat liver homogenate. Ordinate: Concentration of methanol. Abscissa: Incubation time.* ● — ● *1 200 μg of methanol;* ○ —○ *1 200 μg of methanol + boiled homogenate;* ○ — ○ *600 μg of methanol. Experimental conditions as in fig. 4.*

F i g. 7. — *Accumulation of formaldehyde in rat liver homogenate incubated with methanol. Same experiments as in fig. 6. Ordinate: Amount of formaldehyde accumulated. Abscissa: Incubation time. No formaldehyde was accumulated in the boiled homogenate incubated with 1 200 μg of methanol.*

began to decrease after a long incubation period when the utilization of methanol was slow. At that time the utilization of formaldehyde was apparently more rapid than its formation from methanol.

When comparing the curves for the accumulation of formaldehyde and utilization of methanol, note should be taken that the concentration scales for methanol and for formaldehyde in the figures are different. Not more than about 5 to 10 per cent of the hitherto utilized methanol was recovered from the reaction mixture as formaldehyde at any time.

Similar curves for the utilization of methanol and for the accumulation of formaldehyde during this reaction were obtained also with guinea pig liver homogenates. The utilization of methanol, however, was of a somewhat smaller magnitude in these preparations than in the rat liver preparations. The accumulation of formaldehyde in relation to the utilization of methanol was of the same order in the guinea pig and rat liver homogenates.

Effect of Homogenate Concentration

In order to find the most suitable homogenate concentration several series of experiments were performed with variable concentrations of the homogenate. The results from a representative series made with rat liver homogenate incubated aerobically with methanol are presented in figures 8 and 9.

Figure 8 shows the effect of the concentration of the homogenate on the utilization of methanol. From the difference between the amount of methanol added and the methanol concentrations in samples taken just after the addition of the methanol into the homogenates before incubation it is seen that the recovery of methanol decreased with increasing concentrations of homogenate. This effect was most probably due to the binding of methanol to the proteins and other constituents of the homogenate. This decrease in the methanol concentration was most marked in the 30 per cent homogenate, in which the decrease was about 20 per cent of the added amount. It was correspondingly smaller in the more dilute homogenates. In the 10 per cent and 5 per cent homogenates the recovery was practically quantitative.

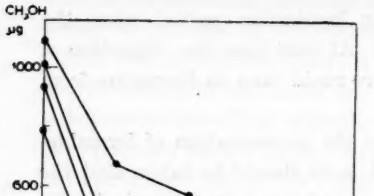

Fig. 8.

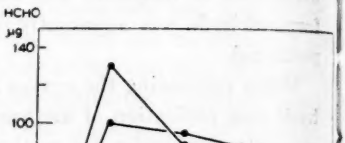

Fig. 9.

F i g. 8— *Utilization of methanol in rat liver homogenates of varying concentrations. Ordinate: Concentration of methanol. Abscissa: Incubation time. 10 ml of homogenate in 0.1 M potassium phosphate buffer, pH 7.4. Concentration of homogenate: Curve 1, 5 per cent; curve 2, 10 per cent; curve 3, 20 per cent; curve 4, 30 per cent. Substrate: 1 100 µg of methanol. Total volume 11 ml. Incubatedat 37°. Gas phase: air.*

F i g. 9. — *Accumulation of formaldehyde in rat liver homogenates of varying concentrations incubated with methanol. Same experiments as in fig. 8. Ordinate: Amount of formaldehyde accumulated. Abscissa: Incubation time.*

The shapes of all the methanol utilization curves in figure 8 are similar, the utilization being rapid during the first 30 minutes and then slowing down. All of the added 1 100 µg of methanol was utilized in the 30 and 20 per cent homogenates during the incubation period of 120 minutes. The utilization in 5 and 10 per cent homogenates was of a proportionately smaller magnitude, about 70 per cent of the added methanol being utilized in the 10 per cent homogenate during 120 minutes.

The accumulation of formaldehyde in these experiments is recorded in figure 9. All the values are corrected for tissue blanks without substrate incubated simultaneously, as were the values for methanol. The most rapid accumulation of formaldehyde occurred during the first 30 minutes and its magnitude was correlated to the concentration of the homogenate. During the

second 30 minutes the formaldehyde concentration increased but little in the 5 and 10 per cent homogenates and was already somewhat decreased in the 20 per cent homogenate. In the 30 per cent homogenate the decrease in the formaldehyde concentration during this time was relatively great, indicating more rapid utilization than formation of formaldehyde. During the second hour of incubation the formaldehyde concentrations still increased somewhat in the two more dilute homogenates and the decrease continued in the two more concentrated homogenates. In the latter all the methanol also was utilized during this time and thus the formation of formaldehyde ceased.

It is difficult to estimate the extent to which the methanol which has become bound to the homogenate can be oxidized to formaldehyde during the incubation. Comparison of the curves for the accumulation of formaldehyde in 30 per cent and 20 per cent homogenates and the corresponding methanol concentration curves suggests that also the bound methanol may be oxidized to formaldehyde at least in part.

The tissue blank values in both the formaldehyde and the methanol determinations were naturally considerably higher in the more concentrated homogenates, and correspondingly the use of direct determinations for formaldehyde and methanol without previous distillation did not give as good results as in the more dilute homogenates. This disadvantageous effect on the formaldehyde and methanol determinations and the binding of considerable amounts of methanol into the concentrated homogenates rendered the quantitative evaluation of the results somewhat difficult, and it was therefore considered more convenient to use 5 or 10 per cent homogenates. With these homogenate concentrations the reactions could still be followed very well and the effect of the binding of methanol to the homogenate was of much smaller magnitude. The tissue blanks also were reasonably small to permit direct determinations of formaldehyde and methanol.

Effect of Substrate Concentration

Time curves for the utilization of methanol in guinea pig liver homogenates using variable initial concentrations of methanol

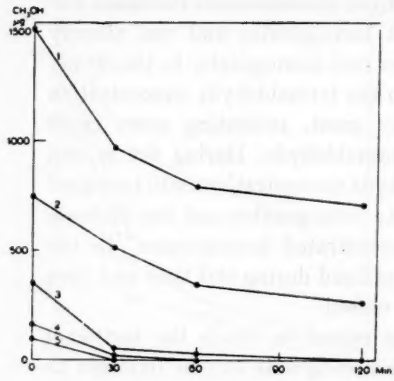

Fig. 10.

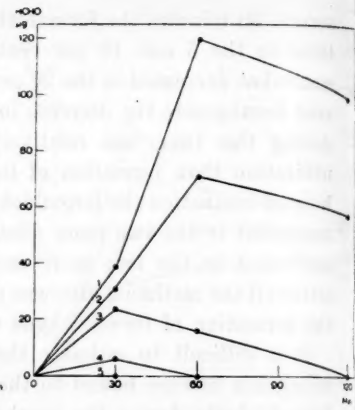

Fig. 11.

Fig. 10. — *Utilization of methanol in guinea pig liver homogenate incubated with varying amounts of methanol. Ordinate: Concentration of methanol. Abscissa: Incubation time. Substrate: Curve 1, 1 600 µg of methanol; curve 2, 800 µg of methanol; curve 3, 400 µg of methanol; curve 4, 250 µg of methanol; curve 5, 125 µg of methanol. 10 ml of 10 per cent homogenate in 0.1 M potassium phosphate buffer pH 7.4. Total volume 11 ml. Incubated at 37°. Gas phase: air.*

Fig. 11. — *Accumulation of formaldehyde in guinea pig liver homogenate incubated with varying amounts of methanol. Same experiments as in fig. 10. Ordinate: Amount of formaldehyde accumulated. Abscissa: Incubation time.*

are presented in figure 10. It is seen that amounts of methanol up to 250 µg, corresponding to a molar concentration of 10^{-3} M in the reaction mixture, were practically totally utilized during the incubation period of 30 minutes. When larger amounts of methanol were added increasing portions of methanol were recovered unchanged after 120 minutes' incubation. Thus the percentile utilization was smaller with higher methanol concentrations.

The accumulation of formaldehyde in these experiments is recorded in figure 11. The maximum accumulation was attained a little later than during the maximum utilization of methanol. At 60 minutes the accumulation of formaldehyde was decreased in all experiments, indicating more rapid utilization than formation of formaldehyde.

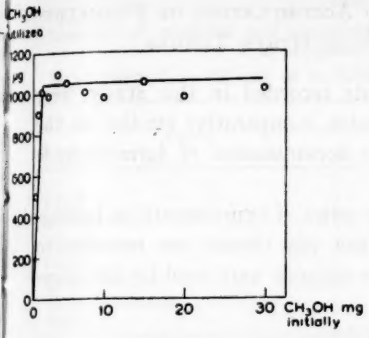

Fig. 12.

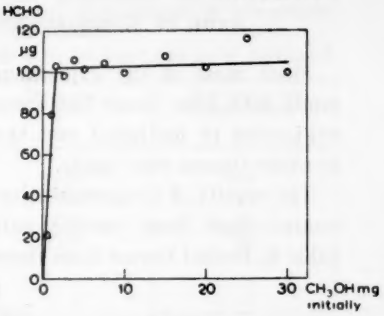

Fig. 13.

Fig. 12. — *Utilization of methanol in rat liver homogenate incubated with varying amounts of methanol. Ordinate: Amount of methanol utilized. Abscissa: Methanol initially. Experimental conditions as in fig. 10. Incubated for 90 min.*

Fig. 13. — *Accumulation of formaldehyde in rat liver homogenates incubated with varying amounts of methanol. Ordinate: Amount of formaldehyde accumulated. Abscissa: Methanol initially. Same experiments as in fig. 12.*

The effect of the initial substrate concentration on the utilization of methanol and the accumulation of formaldehyde in rat liver homogenates is presented in figures 12 and 13. Saturation with substrate was attained already at about 1 000 µg of methanol. The ratio between the amounts of utilized methanol and accumulated formaldehyde was remarkably constant after saturation was attained. In some similar experiments using methanol in amounts up to 100 mg the utilization of methanol and accumulation of formaldehyde began to decrease when more than 25 mg of methanol was used, but this was not seen constantly.

In most of the experiments in this study 1 600 µg of methanol was used as substrate, corresponding to a concentration of 14 mg per 100 ml of reaction mixture or to 160 mg per 100 g tissue in a 10 per cent homogenate. Such concentrations can well be attained in *in vivo* experiments and they were found to be of rather low toxicity.

Utilization of Methanol and Accumulation of Formaldehyde in Homogenates of Other Tissues

Since most of the experiments recorded in this study were made with liver tissue homogenates, comparative studies on the utilization of methanol and the accumulation of formaldehyde in other tissues were made.

The results of a representative series of experiments on homogenates made from various guinea pig tissues are recorded in table 6. Pooled tissues from three animals were used for the prep-

Table 6

Utilization of methanol and accumulation of formaldehyde in homogenates of different guinea pig tissues

10 ml of 10 per cent homogenate in 0.1 M potassium phosphate buffer pH 7.4. Substrate 4 000 µg of methanol. Total volume 11 ml. Incubated for 90 minutes at 37°. Gas phase: air.

Tissue	Methanol utilized µg	Formaldehyde accumulated µg
Liver	1550	136
Kidney	530	20
Spleen	460	28
Lung	365	48
Testis	185	17
Heart muscle	40	5
Skeletal muscle	0	1
Brain	0	0

aration of the homogenates. Three different incubations were simultaneously carried out with each tissue, one incubation being done without added substrate as a control for possible endogenous variations during the incubation. Similar results were obtained in corresponding experiments with the same tissues of other guinea pigs. No significant differences were noted in the relative activities of the tissues of guinea pig and rat, but the absolute values tended to be somewhat higher in rat tissues; however, there were considerable variations in these results. In table 7 are presented the results of a series using rat tissues.

Liver tissue was found to be superior to other tissues in its ability to utilize methanol. The accumulation of formaldehyde was likewise highest in the liver homogenates.

TABLE 7

Utilization of methanol and accumulation of formaldehyde in homogenates of different rat tissues

Experimental conditions as in table 6. Substrate 4 000 μg of methanol.

Tissue	Methanol utilized μg	Formaldehyde accumulated μg
Liver	1890	143
Kidney	680	47
Spleen	310	24
Lung	235	60
Heart muscle	30	8
Skeletal muscle	0	0
Brain	0	0

However, other tissues can also utilize methanol but at a lower rate. Kidney cortex homogenates can utilize methanol, although at a rate which is about one-third of that of liver tissue homogenates. Despite the considerable utilization of methanol, only small amounts of formaldehyde were accumulated in most kidney cortex homogenates, which seems to indicate highly active utilization of formaldehyde in the kidney tissue. Spleen tissue was found to be very active in the utilization of methanol in some preparations. In some animals this utilization was greater than in kidney homogenates, but in others there was only a negligible utilization of methanol. Some methanol was also utilized in homogenates of testis tissue.

The ability of lung tissue homogenates to utilize methanol and the accumulation of formaldehyde varied very much in the different preparations, but some utilization was always present. This activity of lung tissue, which is usually considered rather inert in metabolic reactions, is remarkable. In most preparations the accumulation of formaldehyde in comparison to the amount of methanol utilized was much greater in lung tissue homogenates than in liver and kidney tissue homogenates.

Although muscle tissue is generally regarded as very active metabolically, homogenates from skeletal muscle could not utilize methanol in demonstrable amounts. Heart muscle homogenates had a slight activity in utilizing methanol, and some formaldehyde was accumulated. Homogenates of brain tissue showed no activity

in the utilization of methanol. Neither was any formaldehyde obtained when brain homogenates were incubated with methanol. The brains from a total of 6 guinea pigs and 6 rats were studied in this respect.

Effect of Added Metabolic Cofactors and Inhibitors on the Utilization of Methanol and Accumulation of Formaldehyde in Tissue Homogenates

Effect of Cofactors

The effect of various known metabolic cofactors has been studied in order to obtain information on their possible rôle in these reactions. It was the purpose to study especially if the accumulation of formaldehyde can be prevented by the addition of some cofactor. It had namely been observed in the *in vivo* experiments which are described in the second part of this report that no formaldehyde was accumulated in the blood of animals metabolizing methanol.

Table 8

Utilization of methanol and accumulation of formaldehyde in guinea pig liver homogenates incubated with various cofactors

5 ml of 20 per cent homogenate in 0.1 M potassium phosphate buffer pH 7.4. Substrate 2 000 μg of methanol. Buffer ad 10 ml. Incubated for 90 min. at 37°. Gas phase: air. GSH = 1 mg reduced glutathione; DPN = 1 mg diphosphopyridine nucleotide; ATP = 5 mg adenosine triphosphoric acid, Na salt; cytochrome *c* = 1.5 mg cytochrome *c*; DPT = 0.5 mg diphosphothiamine; Mg^{++} = 5 mg $MgSO_4 \cdot 7\ H_2O$.

Additions	Methanol utilized μg	Formaldehyde accumulated μg
None	820	127
None	950	127
None. Boiled homogenate	4	2
GSH	985	123
DPN	758	142
GSH, DPN	908	124
ATP	784	110
GSH, DPN, ATP	785	98
Cytochrome *c*	857	107
DPT, Mg^{++}	766	140
GSH, DPN, ATP, DPT, Mg^{++}, cytochrome *c*	900	93

In table 8 are presented the results of a series of aerobically conducted experiments in which various metabolic activators and their combinations were added to guinea pig liver homogenates utilizing methanol during an incubation period of 90 minutes. These include diphosphopyridine nucleotide, adenosine triphosphate, diphosphothiamine, reduced glutathione, cytochrome c and magnesium ions. It is seen that none of these activators or their combinations had any distinct effect either on the utilization of methanol or on the accumulation of formaldehyde. The addition of adenosine triphosphate alone or in combinations resulted, it is true, in a slight depression of the accumulation of formaldehyde, but the results were not conclusive.

In the experiments with guinea pig liver homogenate recorded in table 9 the formaldehyde determinations were made 60 and 120 minutes after the beginning of the incubation. The combination of adenosine triphosphate and diphosphopyridine nucleotide caused also here a slight depression of the accumulation of formaldehyde. This effect, however, varied greatly in the different preparations.

TABLE 9

Accumulation of formaldehyde in guinea pig liver homogenates incubated with methanol and various cofactors

10 ml of 10 per cent homogenate in 0.1 M potassium phosphate buffer pH 7.4. Substrate 1 000 µg of methanol. Total volume 12 ml. Incubation temperature 37°. Gas phase: air. Additions: 2.5 mg ATP; 2 mg DPN; 1.25 µg vitamin B_{12}; 100 µg pteroylglutamic acid (PGA).

No.	Additions	Formaldehyde accumulated in	
		60 min. µg	120 min. µg
1	None	27	85
2	None	19	81
3	None	26	82
4	DPN	25	77
5	ATP	20	72
6	DPN, ATP	16	64
7	DPN, ATP	16	70
8	B_{12}	28	85
9	B_{12}	27	93
10	PGA	22	78
11	PGA	25	75
12	PGA, ATP	12	68
13	PGA, ATP	10	64

In experiments 6—13 (table 9) the effect of added pteroylglutamic acid was studied. The addition of pteroylglutamic acid alone resulted in only a slight decrease in the accumulation of formaldehyde. A somewhat more marked decrease was obtained when also adenosine triphosphate was added.

The addition of vitamin B_{12} did not affect the accumulation of formaldehyde in these preparations (experiments 14—15, table 9).

In contrast to the results for liver homogenates, the addition of a mixture of adenosine triphosphate and diphosphopyridine nucleotide to rat or guinea pig lung tissue homogenate approximately doubled the accumulation of formaldehyde. The utilization of methanol increased about 30 per cent. This effect was constant in all the lung tissue homogenates studied. Similar results were obtained with homogenates of guinea pig heart muscle. No effect was seen with homogenates of guinea pig kidney.

Effect of Inhibitors

The effect of various concentrations of cyanide, malonate and monoiodoacetate on the metabolism of methanol was studied in aerobically incubated guinea pig liver homogenates (table 10). The values for the inhibitors refer to their molar concentrations in the reaction mixture.

Cyanide inhibited the utilization of methanol already in rather low concentrations. With 0.0001 M cyanide the inhibition was about 50 per cent. However, cyanide did not totally suppress the utilization of methanol, and some methanol was still utilized when the concentration of cyanide was 0.0050 M. The accumulation of formaldehyde was totally abolished in 0.0005 to 0.0050 M cyanide, and in 0.0001 M cyanide the decrease in the accumulation was about 50 per cent, or the same as the inhibition of the utilization of methanol. The effect of cyanide in abolishing the accumulation of formaldehyde was not necessarily of enzymic nature. Cyanide is namely known to react with formaldehyde to form cyanhydrine, and in dilute formaldehyde solutions this reaction is quantitative and serves also as the principle in Romijn's (1877) method for the quantitative determination of formaldehyde. The absence of an accumulation of formaldehyde when

TABLE 10

Effect of various inhibitors on the utilization of methanol and accumulation of formaldehyde in guinea pig liver homogenate

10 ml of 10 per cent homogenate in 0.1 M potassium phosphate buffer pH 7.4. Substrate 1 600 µg of methanol. Total volume 12 ml. Incubated for 90 minutes at 37°. Gas phase: air.

No.	Inhibitor	Final concentration of inhibitor M	Methanol utilized µg	Inhibition per cent	Formaldehyde accumulated µg	Inhibition per cent
1	None		1110		136	
2	»		1110		138	
3	Iodoacetate	0.001	450	59	60	56
4	»	0.005	260	77	66	52
5	»	0.010	300	73	78	43
6	Malonate	0.005	590	47	89	35
7	»	0.010	590	47	64	53
8	»	0.050	190	83	0	100
9	Cyanide	0.0001	545	51	72	47
10	»	0.0005	228	80	0	100
11	»	0.0010	180	84	0	100
12	»	0.0050	124	89	0	100

larger amounts of cyanide were present may thus also be due to this reaction.

Malonate in a concentration of 0.050 M suppressed the utilization of methanol about 80 per cent and no formaldehyde was found to accumulate. The inhibition was smaller at lower malonate concentrations, and also some formaldehyde was then accumulated.

When 0.010 M monoiodoacetate was employed the utilization of methanol was inhibited about 75 per cent. The percentile inhibition of methanol utilization was practically the same at all the monoiodoacetate concentrations studied, indicating that the inhibition was non-specific and not due to direct inhibition of the methanol-utilizing enzymes. The accumulation of formaldehyde was diminished about 45 per cent in 0.0001 M monoiodoacetate, and this depression was probably to a great extent due to the diminished formation of formaldehyde from methanol. However, monoiodoacetate apparently inhibits somewhat also the enzyme systems responsible for the utilization of formaldehyde since, when its concentration was raised, the accumulation

of formaldehyde increased although the utilization of methanol and therefore also the formation of formaldehyde decreased.

Disulfiram (tetraethylthiuram disulphide, antabuse) is of special interest in studies of the alcohol metabolism. It has been found to inhibit *in vitro* most of the enzymes responsible for the oxidation of aldehydes in the animal organism (KJELDGAARD 1949, RICHERT, VANDERLINDE and WESTERFELD 1950, GRAHAM 1951, NYGAARD and SUMNER 1952), and the administration of disulfiram *in vivo* before or simultaneously with ethanol causes accumulation of acetaldehyde in the blood (LARSEN 1948).

The poor solubility of disulfiram has rendered its use in *in vitro* experiments difficult. Its solubility in water is according to KJELDGAARD (1949) only about 40 μg per ml. In the present experiments, 0.25 mg of disulfiram per ml was homogenized with the tissue. The same technique was used by RICHERT *et al.* (1950), who obtained a marked inhibition of the liver xanthine oxidase and succinoxydase systems. The protein-free filtrate of the homogenate prepared with disulfiram yielded an intense green colour with copper sulphate, indicating that there was disulfiram in the solution (HALD, JACOBSEN and LARSEN 1948).

The effect of disulfiram on the *in vitro* metabolism of methanol was studied in guinea pig liver homogenates (table 11). In these experiments disulfiram had a rather marked depressing effect on the utilization of methanol. The accumulation of formaldehyde

TABLE 11

Effect of disulfiram on the utilization of methanol and accumulation of formaldehyde in homogenates of guinea pig liver tissue

10 ml of 10 per cent homogenate in 0.1 M potassium phosphate buffer pH 7.4. Total volume 11 ml. Substrate 3 200 μg of methanol. Disulfiram homogenized with the tissue. Incubated for 90 minutes at 37°. Gas phase: air.

No.	Additions	Methanol utilized μg	Formaldehyde accumulated μg
1	None	982	106
2	»	1080	93
3	2.5 mg disulfiram	385	84
4	»	352	81
5	»	420	73
6	»	690	88

was depressed by a much smaller percentage, indicating that the enzyme system responsible for the utilization of formaldehyde was also somewhat affected by disulfiram.

Effect of Ethanol on the Utilization of Methanol and Accumulation of Formaldehyde

In view of the many, and in part contradictory, statements in the literature concerning the effect of ethanol in methanol poisoning, it was very interesting to study the effects of ethanol also in *in vitro* systems.

The addition of ethanol to guinea pig and rat liver homogenates incubated aerobically with methanol resulted in a marked decrease both in the utilization of methanol and in the accumulation of formaldehyde. This effect was seen when the molar ratio of ethanol to methanol was 1: 2 or more. With a lower molar ratio no effect or only a negligible one was seen.

In table 12 are presented the results of a series of experiments

Table 12

Effect of ethanol on the utilization of methanol and accumulation of formaldehyde in homogenates of guinea pig liver tissue

10 ml of 10 per cent homogenate in 0.1 M potassium phosphate buffer pH 7.4. Total volume 12 ml. Incubated for 90 minutes at 37°. Gas phase: air. Same homogenate was used in all experiments.

Methanol initially µg	Ethanol µmoles	Methanol utilized µg	Inhibition per cent	Formaldehyde accumulated µg	Inhibition per cent
800 (25 µmoles)	0	442		75	
»	50	304	31	65	15
»	100	201	54	47	37
»	200	115	74	30	60
»	300	92	79	21	72
1600 (50 µmoles)	0	639		115	
»	50	559	13	109	5
»	100	406	37	83	28
»	200	242	62	53	54
»	300	176	73	39	66
3200 (100 µmoles)	0	1051		167	
»	50	787	25	152	9
»	100	541	48	143	14
»	200	408	61	109	34
»	300	326	69	70	58

in which the inhibitory effect of ethanol on the utilization of methanol was studied in aerobically incubated guinea pig liver homogenate. Three concentrations of methanol and four concentrations of ethanol were used. In these experiments the percentage of inhibition of methanol utilization was higher than the percentage of decrease in the accumulation of formaldehyde.

When the values for the utilization of methanol in these experiments are plotted according to LINEWEAVER and BURK (1934) using $\frac{1}{V}$ and $\frac{1}{S}$ as cordinates, where V = reaction velocity (corresponding to the utilization of methanol) and S = substrate concentration, we get the curves shown in figure 14. From these

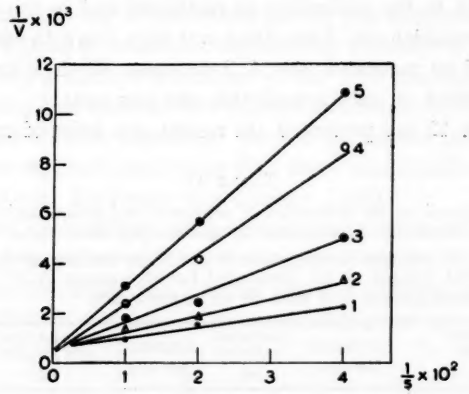

Fig. 14.

F i g. 14. — *Inhibition by ethanol of the utilization of methanol in guinea pig liver homogenates. Data from table 12. Ordinate: Reciprocal of the utilization of methanol in µg × 10⁻³. Abscissa: Reciprocal of the initial concentration of methanol in µmoles × 10⁻². Curve 1, control; curve 2, 50 µmoles of ethanol; curve 3, 100 µmoles of ethanol; curve 4, 200 µmoles of ethanol; curve 5, 300 µmoles of ethanol.*

curves it can be concluded that the inhibition caused by ethanol is most likely of a competitive nature.

Similar results have also been obtained with guinea pig kidney cortex homogenates.

Utilization of Methanol and Accumulation of Formaldehyde in Liver Slices

Many metabolic reactions are known to occur more readily in tissue slices than in homogenates. This is thought to be due to the more intact intracellular organization in the slices. The metabolism of methanol was studied also in liver slices, especially in order to see whether formaldehyde accumulates also in tissue slices when incubated with methanol.

Rat liver slices utilized readily methanol (table 13). About 50

Table 13

Utilization of methanol and accumulation of formaldehyde in rat liver slices incubated with methanol

1 g of slices (wet weight) in 0.1 M potassium phosphate buffer pH 7.4. Total volume 11 ml. Substrate 1 600 μg of methanol. Incubation temperature 37°. Gas phase: air.

No.	Methanol utilized in		Formaldehyde accumulated in	
	30 min. μg	90 min. μg	30 min. μg	90 min. μg
1	386	838	17	33
2	285	500	20	25
3	364	870	17	34
4	415	810	24	30

per cent of the added 1 600 μg of methanol was utilized during the incubation for 90 minutes at 37°. Formaldehyde accumulated also in the rat liver slices utilizing methanol. The magnitude of this accumulation, however, was smaller than in the corresponding experiments with liver homogenates. After incubation for 90 minutes about 5 per cent of the methanol utilized up to that time was accumulated as formaldehyde.

Corresponding experiments were made also with slices from guinea pig liver tissue (table 14). As in the case of homogenates, the utilization of methanol in guinea pig tissue preparations was of a smaller magnitude than that in the corresponding rat tissue preparations. During incubation for 90 minutes only 20 per cent of the added 1 600 μg was utilized in slices from guinea pig liver; the utilization in rat liver slices was 50 per cent. The accumulation

TABLE 14

Utilization of methanol and accumulation of formaldehyde in guinea pig liver slices incubated with methanol

1 g of slices (wet weight) in 0.1 M potassium phosphate buffer pH 7.4. Total volume 11 ml. Substrate 1 600 µg of methanol. Incubated at 37° for 90 minutes. Gas phase: air.

No.	Methanol utilized µg	Formaldehyde accumulated µg
1	286	9
2	438	6
3	352	8
4	450	15

of formaldehyde was negligible in these experiments with guinea pig liver, but traces of formaldehyde were always found.

The dependence of the utilization of methanol and the accumulation of formaldehyde on the pH of the reaction mixture was studied also in guinea pig liver slices (figures 15 and 16). In these experiments the incubation time was 180 minutes and the sub-

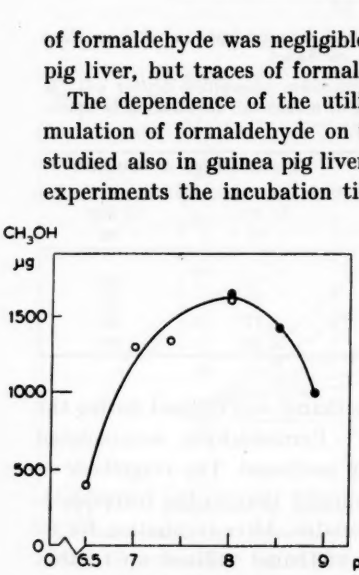

Fig. 15.

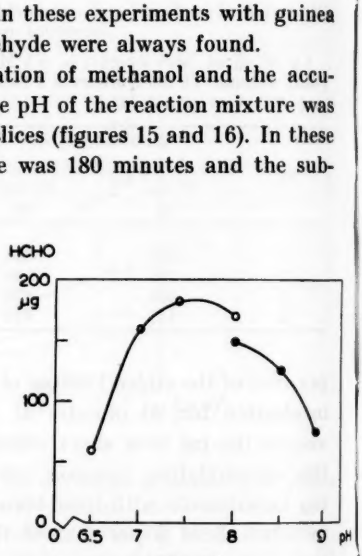

Fig. 16.

F i g. 15. — *Effect of pH on the utilization of methanol in guinea pig liver slices. Ordinate: Amount of methanol utilized. Abscissa: pH of buffer. 2 g of slices (wet weight) in 10 ml of 0.1 M buffer.* ○ = *Sörensen's phosphate buffer;* ● = *pyrophosphate buffer. Substrate: 3 200 µg of methanol. Total volume 11 ml. Incubated for 180 minutes at 37°. Gas phase: air.*

F i g. 16. — *Effect of pH on the accumulation of formaldehyde in guinea pig liver slices incubated with methanol. Same experiments as in fig. 15. Ordinate: Amount of formaldehyde accumulated. Abscissa: pH of buffer.*

strate amount 3 200 μg of methanol. Under these conditions considerable amounts of formaldehyde were accumulated. The pH curve for the accumulation of formaldehyde was quite similar to the corresponding curve obtained for liver homogenates. As in the experiments with the homogenate technique, a small difference between ordinary phosphate and pyrophosphate buffers was noted also here. The pH dependence of the utilization of methanol in liver slices was also similar to that in liver homogenates.

The ageing of the liver homogenate resulted in an increased accumulation of formaldehyde without any effect on the utilization of methanol. Corresponding studies were also made by the tissue slice technique, where the tissue used was stored for various lengths of time before the preparation of slices. This effect of the time interval between the killing of the animal and the beginning of the incubation was investigated using guinea pig liver tissue. The tissue was stored in a refrigerator at + 2°. After various time intervals a part of the tissue was sliced and the slices were immediately used for the experiments. The time intervals in table 15 refer to the time between the killing of the

TABLE 15

Effect of ageing of the tissue on the utilization of methanol and accumulation of formaldehyde in slices from guinea pig liver tissue

Tissue stored at +2° and sliced after various time intervals. 2 g of slices (wet weight) in 10 ml 0.1 M Sörensen's phosphate buffer pH 7.4. Total volume 11 ml. Substrate 3 200 μg of methanol. Incubated for 180 minutes at 37°. Gas phase: air.

No.	Tissue ageing time min.	Methanol utilized μg	Formaldehyde accumulated μg
1	25	1910	260
2	90	1620	290
3	235	1730	405
4	15	1870	235
5	120	1950	300
6	300	1700	445

animal and the beginning of the incubation. The livers of two animals were used, experiments 1—3 being made with the one liver and experiments 4—6 with the other.

The ageing of the tissue increased the accumulation of formal-

dehyde from methanol in these experiments. This increase was 55 per cent in the first group, in which the tissue was stored for 235 minutes, and 90 per cent in the second group, in which the storage time was 300 minutes. In the utilization of methanol, on the other hand, there was no clear correlation to the storage time of the tissue, all the values being of about the same magnitude.

Tissue blanks without added substrate were run for each experiment. The values for both formaldehyde and methanol in these blanks increased with increase in the storage time of the tissue. However, after incubation these values were of about the same magnitude both in aged and fresh preparations, indicating that the substances responsible for the greater blank values in the unincubated samples were apparently metabolized during the incubation. All these blank values were so small that they had no effect upon the final results.

Utilization of Formaldehyde in Tissue Homogenates

The accumulation of formaldehyde during the utilization of methanol in animal tissue preparations was studied in the preceding sections of this investigation. This accumulation of formaldehyde indicates that the utilization of formaldehyde apparently is a rate-limiting reaction in these preparations. The experiments recorded in this section were made in order to study the utilization of added formaldehyde in homogenates of animal tissue.

In figure 17, results are presented for a representative series of experiments where 400 μg and 100 μg of formaldehyde were incubated in air with a 10 per cent homogenate of rat liver tissue. The concentration of formaldehyde in the incubation mixture was determined after various time intervals. It is seen that formaldehyde was readily utilized in these preparations. The utilization was most rapid during the first 30 minutes of incubation and then slowed down. At 120 minutes about 75 per cent of the formaldehyde present at 0 minute had disappeared in the experiments in which 400 μg were added. The corresponding disappearence was 90 per cent when 100 μg were added. The utilization of formaldehyde was considerably slower when the added amount was smaller.

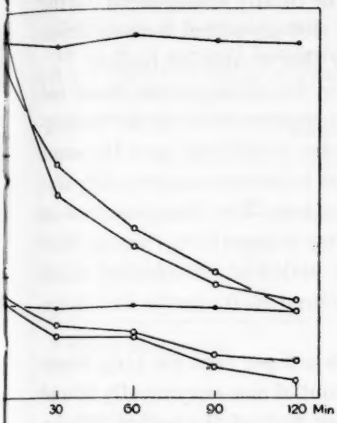

Fig. 17.

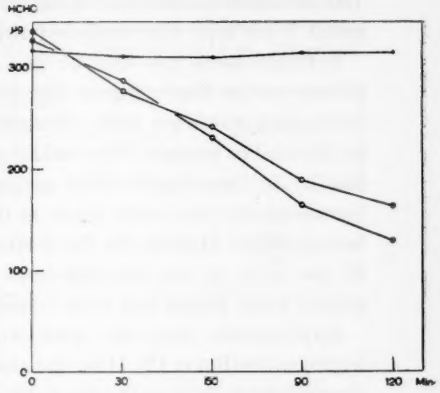

Fig. 18.

Fig. 17. — *Utilization of formaldehyde in rat liver homogenate. Ordinate: Concentration of formaldehyde. Abscissa: Incubation time. 10 ml of 10 per cent homogenate in 0.1 M potassium phosphate buffer pH 7.4. Total volume 11 ml. Substrate: 400 µg and 100 µg of formaldehyde. Smaller circles indicate boiled homogenate. Incubated for 120 minutes at 37°. Gas phase: air.*
Fig. 18. — *Utilization of formaldehyde in rat kidney cortex homogenate. Ordinate: Concentration of formaldehyde. Abscissa: Incubation time. 5 per cent homogenate. Substrate: 400 µg of formaldehyde. Experimental conditions otherwise as in fig. 17.*

Boiled homogenates did not utilize formaldehyde. This can be regarded as an indication of the enzymic nature of the disappearance of formaldehyde in the unboiled homogenates. A fraction of formaldehyde is apparently bound to the protein and other constituents of the homogenates, as is seen from the difference between the amounts of added formaldehyde and formaldehyde recovered at 0 minute. However, this difference was of about the same magnitude in the boiled and unboiled homogenates of the same concentration and the results can therefore be regarded as comparable.

In another series using rat liver homogenates where formaldehyde was incubated with a 10 per cent rat liver homogenate and determinations were made only at 0 and 180 minutes it was found

that the added 100 μg of formaldehyde totally disappeared during 180 minutes' incubation in air. Of 400 μg added formaldehyde about 5 per cent was recovered after this incubation period.

Formaldehyde can also be utilized by homogenates from rat kidney cortex tissue (figure 18). The concentration of the homogenate used was 5 per cent; otherwise the conditions were the same as above. The amount of formaldehyde bound non-enzymically was smaller in these more dilute preparations. The disappearance of formaldehyde was more linear in these preparations than in liver homogenates. During the incubation period of 120 minutes about 25 per cent of the formaldehyde found at 0 minute had disappeared when 400 μg had been added.

Experiments were also made with a 5 per cent rat lung tissue homogenate (figure 19). Here the amount of non-enzymically bound formaldehyde was only about 10 per cent of the added 100 μg. During the incubation of 120 minutes about 50 per cent of the formaldehyde had disappeared.

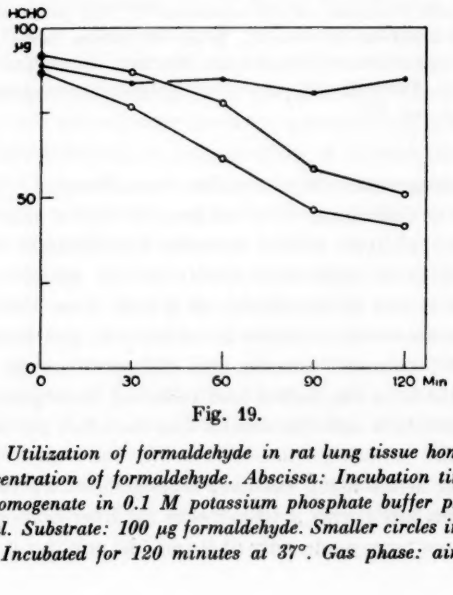

Fig. 19.

Fig. 19. — *Utilization of formaldehyde in rat lung tissue homogenate. Ordinate: Concentration of formaldehyde. Abscissa: Incubation time. 10 ml of 5 per cent homogenate in 0.1 M potassium phosphate buffer pH 7.4. Total volume 11 ml. Substrate: 100 μg formaldehyde. Smaller circles indicate boiled homogenate. Incubated for 120 minutes at 37°. Gas phase: air.*

TABLE 16

Utilization of formaldehyde in homogenates of guinea pig liver tissue

10 ml of 10 per cent homogenate in 0.1 M potassium phosphate buffer pH 7.4. Substrate 400 μg of formaldehyde. Total volume 11 ml. Gas phase: air. Incubation temperature 37°.

No.	Formaldehyde recovered		Difference μg
	0 min. μg	90 min. μg	
1	295	96	199
2	280	100	180
3	265	80	185
4[1]	270	255	15

[1] Boiled homogenate

In table 16 are presented the results of experiments with homogenates from guinea pig liver tissue. A 10 per cent homogenate was used and the conditions were the same as in experiments with rat liver. The ability of guinea pig liver homogenates to utilize formaldehyde was of the same magnitude as that of rat liver homogenates.

To study the effect of added methanol and ethanol on the utilization of formaldehyde the experiments recorded in table 17 were made. In these experiments methanol or ethanol was added with the formaldehyde when incubation was started and the utilizations of formaldehyde and methanol were determined after 90 minutes. The addition of 50 or 100 μmoles of ethanol had no effect on the utilization of formaldehyde. When corresponding amounts of methanol were added there was a definite decrease of the difference in the formaldehyde concentrations at 0 and 90 minutes. This was at least partly due to the formaldehyde formed from methanol during the incubation, as a result of which much more formaldehyde was actually utilized than is evident from the figures in table 17.

The utilization of methanol should decrease when formaldehyde is added if formaldehyde is in fact an intermediary product in this reaction. From table 17 it is seen that the addition of 500 μg of formaldehyde diminished the utilization of 1 600 μg of methanol by about 50 per cent.

The effect of disulfiram on the utilization of formaldehyde in

TABLE 17

Effect of added methanol and ethanol on the utilization of formaldehyde in guinea pig liver homogenates

10 ml of 10 per cent homogenate in 0.1 M potassium phospate buffer pH 7.4. Total volume 12 ml. Incubated at 37°. Gas phase: air.

No.	Substrate	Formaldehyde recovered		Difference μg	Methanol utilized μg
		0 min. μg	90 min. μg		
1	500 μg HCHO	356	89	−267	
2	500 μg HCHO	380	98	−282	
3	1600 μg CH_3OH	2	95	+ 93	650
4	3200 μg CH_3OH	2	108	+106	882
5	500 μg HCHO + 1600 μg CH_3OH	390	216	−174	226
6	500 μg HCHO + 3200 μg CH_3OH	427	227	−200	602
7	500 μg HCHO + 2300 μg C_2H_5OH	384	107	−277	
8	500 μg HCHO + 4600 μg C_2H_5OH	400	145	−255	

guinea pig liver homogenates is presented in table 18. Although the utilization of formaldehyde was smaller in the homogenates incubated with disulfiram, this difference can hardly be considered significant.

TABLE 18

Effect of disulfiram on the utilization of formaldehyde in guinea pig liver homogenates

10 ml of 10 per cent homogenate in 0.1 M potassium phosphate buffer pH 7.4. Substrate 500 μg of formaldehyde. Total volume 11 ml. Disulfiram was homogenized with the tissue. Gas phase: air.

No.	Additions	Formaldehyde recovered		Difference μg
		0 min. μg	90 min. μg	
1	None	356	89	267
2	»	380	98	282
3	2.5 mg disulfiram	400	170	230
4	»	356	110	246
5	»	396	136	260
6	»	370	130	240

DISCUSSION

The results of the present experiments demonstrate clearly that homogenates of rat and guinea pig liver can readily utilize methanol. The enzymic nature of this utilization was shown by the failure of the boiled homogenates to utilize methanol, and it had also other characteristics of enzymic reactions. Formaldehyde was also found to be accumulated in the reaction mixture during the utilization of methanol. The accumulation of formaldehyde ran remarkably parallel with the utilization of methanol in all experiments. It would thus be possible to obtain some information of the utilization of methanol by following only the accumulation of formaldehyde. The amount of formaldehyde recovered at any time from the reaction mixture has been maximally 10—20 per cent of the methanol utilized up to that time. If we assume that all the methanol was utilized via formaldehyde as an intermediate, the greatest part of the formed formaldehyde must have been metabolized further in the homogenates. Some of it was probably also bound non-enzymically to the homogenate.

There was considerable variation in the activity of homogenates prepared at different times, especially in the amount of formaldehyde accumulated as compared to the amount of utilized methanol. This variation was probably at least in part due to greater lability of the enzyme systems responsible for the utilization of formaldehyde in comparison to the enzymes which utilize methanol. This was demonstrated in the experiments in which the homogenates were aged before incubation, which increased markedly the accumulation of formaldehyde without any clear effect on the utilization of methanol. A partial inactivation of cofactors necessary for the utilization of formaldehyde may also be responsible for this increased accumulation of formaldehyde in the aged homogenates.

Liver tissue homogenates were most active in the utilization of methanol. However, other tissues could also utilize methanol. Homogenates of the kidney, spleen and lung tissues had considerable activities, and homogenates of testis and heart muscle tissues also had some activity. Homogenates of brain and skeletal muscle tissues did not utilize methanol. This inactivity of brain

tissue is remarkable in view of the special neurotoxicity of methanol. The relative capacities of methanol utilization of different tissues are well in accordance with the results of BARTLETT (1950 a), who studied the oxidation of isotope-labelled methanol into carbon dioxide in slices of rat liver, kidney, heart muscle, diaphragm and brain tissues. Slices of rat lung tissue are able to oxidize ethanol (MASORO and ABRAMOWITCH 1953, MASORO, ABRAMOWITCH and BIRCHARD 1953) but slices of rat spleen tissue have had no ethanol oxidizing activity (LELOIR and MUÑOZ 1938). In the present work, rat tissues utilized generally somewhat more methanol than guinea pig tissues, although the relative capacities of different tissues were same in both species. NYBERG, SCHUBERTH and ÄNGGÅRD (1953) found no measurable quantities of alcohol dehydrogenase in guinea pig liver and only very small quantities in rat liver, which may partly explain these differences.

The failure of the various cofactors to influence the utilization of methanol and the accumulation of formaldehyde in liver homogenates indicates that these homogenates contain enough of these cofactors for the utilization of methanol and formaldehyde. Further studies with dialysed and more purified preparations are needed to settle which cofactors are in fact directly concerned with these reactions. The decrease in the accumulation of formaldehyde in liver homogenates caused by the addition of adenosine triphosphate and pteroylglutamic acid may be due to the stimulation by adenosinetriphosphate of the transformation of pteroylglutamic acid to tetrahydropteroylglutamic acid, which can transfer hydroxymethyl groups to various acceptors (ALEXANDER and GREENBERG 1955). Lung and heart muscle homogenates, on the other hand, contained apparently not enough of endogenous diphosphopyridine nucleotide and adenosine triphosphate for maximum utilization of methanol. In similar experiments with rat liver slices using ethanol as substrate the addition of diphosphopyridine nucleotide also had no effect on the utilization of ethanol (KERNER and WESTERFELD 1950).

Ethanol caused a marked inhibition of the utilization of methanol in liver and kidney homogenates. When the data of experiments with liver homogenate were plotted according to LINEWEAVER and BURK (1934) it could be concluded that the inhibition

was competitive. These results are in agreement with those of the experiments of ZATMAN (1946) and BARTLETT (1950 b), in which the amount of utilized methanol was determined indirectly. More ethanol, however, was needed in the present experiments to obtain a demonstrable inhibition. This is probably due to the different experimental techniques and tissue preparations used. The accumulation of formaldehyde was also diminished when ethanol was added, but the percentile inhibition was lower than that of the utilization of methanol. Ethanol had, however, no effect on the utilization of added formaldehyde in liver homogenates. The most natural explanation for the effect of ethanol on the utilization of methanol would be a competitive inhibition of the oxidation of methanol by alcohol dehydrogenase, as has been suggested by ZATMAN (1946).

The inhibitory effects of cyanide, monoiodoacetate and malonate on the metabolism of methanol were probably mostly secondary to the inhibition of other metabolic reactions and no definite conclusions can be drawn from them concerning the metabolism of methanol. The effect of cyanide was apparently due to the inhibition of cytochrome oxidase, which inhibits also the entire aerobic metabolism almost totally. The results for the utilization of methanol and the accumulation of formaldehyde in the presence of cyanide were quite similar to the values obtained under anaerobic conditions. Of the enzymes which can oxidize methanol, catalase is readily inhibited by cyanide (WARBURG 1923). Liver alcohol dehydrogenase is not inhibited by cyanide under anaerobic conditions (LUTWAK-MANN 1938), which indicates that the inhibition seen in aerobic experiments is not due to the direct blocking of the alcohol dehydrogenase. The percentile inhibition of methanol utilization caused by the special sulphydryl group inhibitor monoiodoacetate, was practically the same at all the iodoacetate concentrations indicating that the inhibition was not direct. Both liver alcohol dehydrogenase and catalase are known to be insensitive to monoiodoacetate (LUTWAK-MANN 1938, BARRON and SINGER 1945). The apparent inhibition of the utilization of formaldehyde by monoiodoacetate may be specifically due to inhibition of the glutathione-dependent formaldehyde dehydrogenase of STRITTMATTER and BALL (1955). The dependence

of the utilization of methanol on the general tissue metabolism is also seen in the partial inhibition caused by malonate, which is known to inhibit competitively the succinodehydrogenase system (QUASTEL and WOOLDRIDGE 1928) and thus also the citric acid cycle.

LELOIR and MUÑOZ (1938) have obtained similar results in their studies on the effect of various inhibitors on the oxidation of ethanol in rat liver slices. Cyanide greatly diminished the oxidation of ethanol but did not stop it completely. Monoiodoacetate and malonate were also inhibitory.

Disulfiram inhibited markedly the utilization of methanol in liver homogenates, but caused only a slight depression of the utilization of formaldehyde. In earlier investigations disulfiram has been found to inhibit *in vitro* the enzymes concerned with the oxidation of acetaldehyde. According to KJELDGAARD (1949) disulfiram inhibits the action of the liver flavoprotein aldehyde oxidase already in very low concentrations. Liver xanthine oxidase (RICHERT, VANDERLINDE and WESTERFELD (1950), glyceraldehyde-3-phosphate dehydrogenase (NYGAARD and SUMNER 1952) and the diphosphopyridine nucleotide-dependent aldehyde dehydrogenase (GRAHAM 1951) are also inhibited by disulfiram. The inhibitory action of disulfiram, however, is not limited to these enzymes but it also causes a more marked inhibition of the endogenous respiration of rat liver homogenates than would be due to the inhibition of only the aforementioned enzymes (EDWARDS 1949, RICHERT *et. al.* 1950). It has also been reported to inhibit the succinodehydrogenase system (RICHERT *et. al.* 1950, GRAHAM 1951, GAL and GREENBERG 1954, MUSTAKALLIO and SAIKKONEN 1955). The inhibition of the utilization of methanol observed in the present experiments may be explained by a general metabolic inhibition, but direct inhibition of the enzymes utilizing methanol is also possible. The dehydrogenation of ethanol, citrate and succinate is markedly depressed by disulfiram in rat liver homogenates (GRAHAM 1951, GAL and GREENBERG 1954). According to KJELDGAARD (1949), crystalline horse liver alcohol dehydrogenase is not sensitive to disulfiram but the disulfiram concentrations used by him were very small.

The slight inhibition of the utilization of formaldehyde found

in the present experiments indicates that the above mentioned acetaldehyde oxidizing enzymes, which are sensitive to disulfiram, apparently play no great rôle in the metabolism of formaldehyde although they are known to oxidize also formaldehyde. We have no knowledge of the possible effect of disulfiram on the specific formaldehyde dehydrogenase of STRITTMATTER and BALL (1955).

Slices and homogenates were about equally active in the utilization of methanol, which indicates that it is not to any great extent dependent on the integrity of the intracellular structures. Less formaldehyde was, however, accumulated in the reaction mixture when slices were used. LELOIR and MUÑOZ (1936) have made similar observations concerning the metabolism of ethanol. They found that appreciable quantities of acetaldehyde were accumulated in liver brei metabolizing ethanol, but not in liver slices.

Homogenates of liver, kidney and lung tissues were able to utilize added formaldehyde enzymically. A part of the added formaldehyde was fixed non-enzymically to the homogenate. This non-enzymatic fixation of formaldehyde was of the same magnitude also in the control experiments in which boiled homogenates were used. No additional disappearence of formaldehyde was seen in the boiled homogenates during the incubation, which indicates the enzymic nature of the utilization in unboiled homogenates. In the experiments of BERNHEIM (1950) and MILLER (1953) formaldehyde was slowly oxidized in rat liver suspensions after an initial inhibition of the oxygen uptake. In the present experiments the utilization of formaldehyde was most rapid during the first 30 minutes of incubation and then slowed down.

METABOLISM OF METHANOL *IN VIVO* IN RABBITS

Rabbits were used as experimental animals in the *in vivo* studies, The elimination from the blood of small amounts of methanol administered perorally or intravenously was followed and the elimination rate of methanol from the blood was determined. The effects of ethanol and disulfiram on the elimination of methanol from blood were also investigated. The possible formaldehyde content of blood was determined in all these experiments to find out if formaldehyde

can be demonstrated in blood after the administration of methanol. In some of the experiments the urinary excretion of methanol, formic acid and formaldehyde was also determined.

Elimination of Methanol from Blood

Since information in the literature concerning the elimination of methanol from the blood is very limited and also rather conflicting it was considered advisable to study first the elimination of methanol from the blood of normal rabbits.

Methanol was given *per os* by a stomach tube to 17 rabbits in doses of 0.8 to 3.8 g per kg of body weight. The methanol was first diluted with water to a concentration of 10 to 20 per cent. These methanol concentrations apparently caused no appreciable irritation of the stomach because no vomiting occurred in any of the cases. Some typical curves for the disappearance of methanol from the blood are presented in figures 20 and 21.

In the experiments recorded in figure 20 the dosage of methanol *per os* varied from 3.8 to 2.4 g per kg of body weight. It is seen

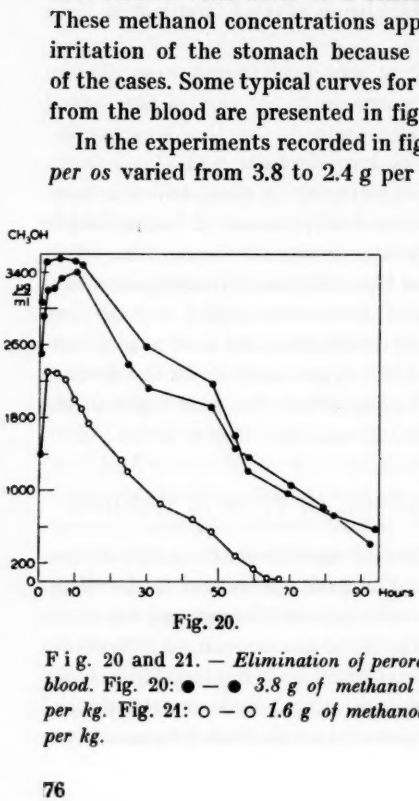

Fig. 20.

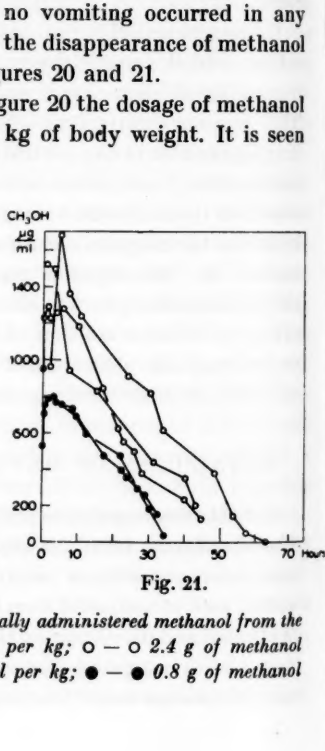

Fig. 21.

Fig. 20 and 21. — *Elimination of perorally administered methanol from the blood.* Fig. 20: ● — ● *3.8 g of methanol per kg;* ○ — ○ *2.4 g of methanol per kg.* Fig. 21: ○ — ○ *1.6 g of methanol per kg;* ● — ● *0.8 g of methanol per kg.*

that the time from the administration of methanol to the full diffusion equilibrium varied from 5 to 10 hours, being apparently longer with the higher methanol concentrations. The curves then began to decline, the methanol concentration in the blood decreasing at first more rapidly, and then more slowly at the lower concentrations. The shape of the curves is therefore hyperbolic. The elimination rate at the higher blood concentrations was about 0.00110—0.00080 mg per ml per minute and at the lower concentrations 0.00035—0.00050 mg.

In the experiments presented in figure 21 methanol doses from 0.8 to 1.6 g per kg of body weight were administered *per os*. The hyperbolic shape of the curves was still seen when 1.6 g per kg were administered, the elimination rate being at the higher blood concentrations 0.00135—0.00075 mg per ml per minute and at the lower concentration 0.00042—0.00035 mg. With a dose of 0.8 g per kg the deviation of the curve from linearity was very small in most cases and the curves may very well be treated as linear. In these experiments the elimination rate thus was constant and about 0.00040 mg per ml per minute in most cases.

The shape of the curves varied considerably during the first 10 hours after administration. These variations were in all probability due to differences and disorders in resorption caused by the relatively high concentration of the administered alcohol.

To avoid these variable resorption factors affecting the elimination curves, experiments were also made where the methanol diluted with physiological sodium chloride solution was administered intravenously to altogether 12 rabbits. The doses used were 0.8 g and 0.2 g per kg. Some of the results are presented in figures 22 and 23. The deviation from linearity is not notable at these low dosage levels. The elimination rate was about 0.00046 and 0.00038 kg per ml per minute at dose levels 0.2 g and 0.8 g per kg, respectively. No differences are observed in the curves when the methanol was administered either perorally or intravenously, with the exception of the aforementioned variation in resorption during the first 5 to 10 hours.

Formaldehyde determinations were made on all blood samples but no values above blanks were obtained in any of these experiments, indicating that no formaldehyde accumulated in the

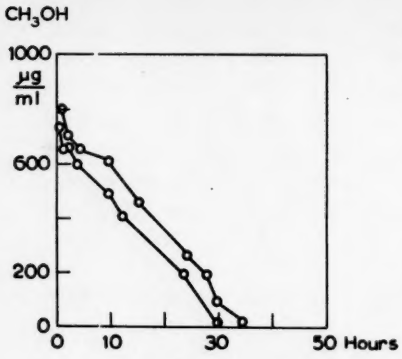

Fig. 22.

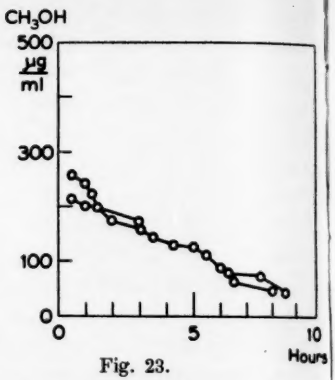

Fig. 23.

Fig. 22 and 23. — *Elimination of intravenously administered methanol from the blood.* Fig. *22: 0.8.g of methanol per kg.* Fig. *23: 0.2 g of methanol per kg.*

blood after the administration of methanol in doses up to 3.8 g per kg. An experiment was also made in which 5.0 g of methanol per kg of body weight was administered *per os* and the blood analysed for formaldehyde 5, 10, 15, 20 and 30 hours later. No formaldehyde could be demonstrated, however, in any of the samples.

The transitory increase in the blood concentration of methanol seen 4 to 6 hours after the administration of methanol to rabbits by BILDSTEN (1924) and BERNHARD and GOLDBERG (1933) was not present in these experiments. The small rises which were found at this time in some curves were of about the same magnitude as the fluctuations generally seen in the curves.

In table 19 are collected all the observed values for the elimination rate of methanol from the blood of rabbits at dosage levels of 0.8 g per kg and lower. These values correspond to the constant β in the following well known equation of WIDMARK (1922):

$$A = p\ r\ (c_t + \beta t),$$

where A=administered amount of alcohol, in g, p=body weight, in kg, r=individual factor for reduction of body weight to the part which is in equilibrium with blood in regard to the distribution of alcohol, c_t=concentration of alcohol at time t, in mg per g,

TABLE 19

Elimination rate of methanol from the blood in rabbits

The values for β are computed by the method of least squares.

Rabbit No.	Dose of methanol g per kg	β g per ml per minute	Rabbit No.	Dose of methanol g per kg	β g per ml per minute
1	0.8 per os	0.000368	5	0.8 iv.	0.000380
2	0.8 per os	0.000393	6	0.8 iv.	0.000372
3	0.8 per os	0.000429	24	0.8 iv.	0.000426
4	0.8 per os	0.000443	28	0.8 iv.	0.000411
13	0.8 per os	0.000496	35	0.8 iv.	0.000388
16	0.8 per os	0.000373	44	0.8 iv.	0.000462
17	0.8 per os	0.000468	48	0.8 iv.	0.000453
8	0.2 iv.	0.000479	49	0.8 iv.	0.000388
9	0.2 iv.	0.000369	51	0.8 iv.	0.000457
12	0.2 iv.	0.000392			
52	0.2 iv.	0.000420			

t=time after administration, β=constant of the rate at which alcohol disappears from the blood after equilibrium, in mg per g per minute. In this study the blood methanol concentrations are not calculated according to WIDMARK as mg per g but as mg per ml of blood.

It is seen from table 19 that the elimination rate at this low dosage level was 0.00042 ± 0.00004 mg per ml per minute. No differences between peroral and intravenous administration were observed. When the blood methanol concentration was 2500 to 800 μg per ml the elimination rate varied between 0.00200 and 0.00070 mg per ml per minute, being on an average 0.00120 (9 observations).

EFFECT OF ETHANOL ON THE ELIMINATION OF METHANOL FROM BLOOD

The effect of ethanol on the elimination of methanol was studied in 13 rabbits.

The results of experiments in which a mixture of ethanol and methanol was administered *per os* are registered in figure 24. When the dose of methanol was 1.6 g per kg and the ethanol was administered simultaneously in doses from 0 to 1.6 g per kg it is seen that after some 15 hours the elimination rates were equal in all the experiments. During the resorptive phase there were wide fluctuations of the values, especially in the experiments where

Fig. 24. — *Effect of ethanol on the elimination of methanol from the blood when both are administered perorally.* ○—○ *1.6 g of methanol per kg + 1.6 g of ethanol per kg;* ⊕—⊕ *1.6 g of methanol per kg + 1.2 g of ethanol per kg;* ⊙—⊙ *1.6 g of methanol per kg + 0.8 g of ethanol per kg;* ●—● *1.6 g of methanol per kg.*

both ethanol and methanol were administered. However, it is readily seen that when ethanol also was administered the methanol concentration tended to show high values for a longer time before the decline set in. Consequently the blood concentration of methanol in the latter part of the experiments was higher in the ethanol-treated animals and was correlated to the amount of administered ethanol.

In order to avoid the resorption factors which influence the shape of the first part of the elimination curves the alcohols were also administered intravenously (figure 25). To facilitate injection the dose of methanol was rather small in these experiments, namely 0.2 and 0.4 g per kg of body weight. It was administered simultaneously with 0.8 g of ethanol per kg in 10 ml of physiological saline.

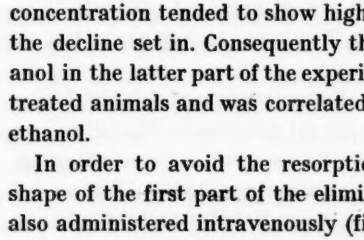

Fig. 25. — *Effect of ethanol on the elimination of methanol from the blood when both are administered intravenously.* ●—● *(upper curve) 0.4 g of methanol per kg + 0.8 g of ethanol per kg;* ○—○ *0.2 g of methanol per kg + 0.8 g of ethanol per kg;* ●—● *(lower curve) 0.2 g of methanol per kg.*

A control curve for 0.2 g of methanol given intravenously without ethanol is also presented for comparison. The retarding effect of ethanol on the elimination of methanol is seen very clearly at both dosage levels of methanol. The duration of this effect was about the same in both cases, i.e., about 4 to 5 hours. After this time the elimination rate was identical in both experiments and the same as in the experiments made without ethanol.

If the ethanol was administrated 10 hours after the administration of methanol, the retarding effect came out very distinctly. In the experiment presented in figure 26, 0.8 g of methanol per kg was administered intravenously, followed 10 hours later by 2.0 g ethanol per kg *per os*. During the following 15 hours the elimination of methanol was very low, but thereafter the decline of the methanol concentration in the blood continued at the same rate as before the administration of ethanol.

It was not possible to demonstrate any formaldehyde in these blood samples.

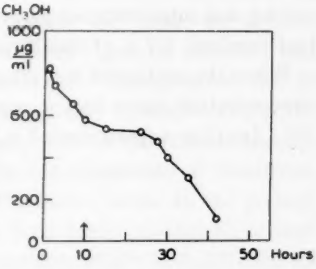

Fig. 26. — *Effect of ethanol on the elimination of methanol from the blood when administered 10 hours after methanol. Dose: 0.8 g of methanol per kg intravenously, followed 10 hours later by 2.0 g of ethanol per kg perorally.*

EFFECT OF DISULFIRAM ON THE ELIMINATION OF METHANOL[1] FROM BLOOD

The effect of disulfiram was studied in 12 rabbits. In the experiments recorded in figure 27, 0.7 g of disulfiram per kg was given *per os* one hour before the administration of methanol. The dose of methanol was 0.8 g per kg perorally. The administration of disulfiram retarded the elimination of methanol from the blood very distinctly, and the blood methanol concentration curve which

[1] A preliminary report of some of these results has been published elsewhere (Koivusalo 1956).

normally at this dosage level was practically linear became slightly hyperbolic. Following the administration of disulfiram, methanol was still detectable in the blood 100 hours after administration, although normally the blood was free from methanol about 35—45 hours after administration of this amount of methanol.

Disulfiram apparently affects also the resorption of methanol. The methanol concentrations in the blood some three hours after the administration of methanol were clearly higher in the disulfiram-treated rabbits. This may be due to the fact that there was still much unresorbed disulfiram left in the digestive tract when an about 15 per cent solution of methanol was given. When methanol was given intravenously this effect was not seen.

When 0.8 g of disulfiram per kg was given *per os* without the subsequent administration of methanol, no effect was seen in the blood methanol determinations, which did not differ significantly from the blank values.

Results similar to the above were obtained when 1.6 g of methanol per kg was administered perorally to rabbits which 1 hour earlier had received 0.7 g of disulfiram per kg (figure 28).

When the methanol was given intravenously the blood methanol concentration curve had a somewhat different appearance (figure 29). In this experiment 1.0 g of disulfiram per kg had been

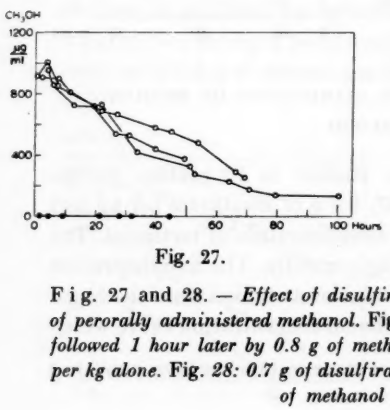

Fig. 27.

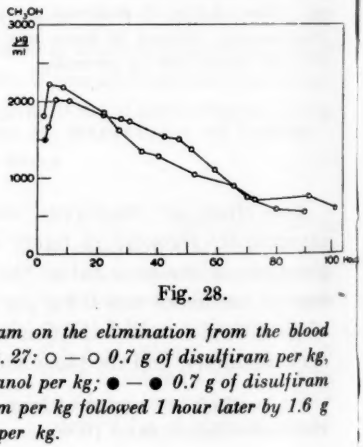

Fig. 28.

Fig. 27 and 28. — *Effect of disulfiram on the elimination from the blood of perorally administered methanol.* Fig. *27:* ○ — ○ *0.7 g of disulfiram per kg, followed 1 hour later by 0.8 g of methanol per kg;* ● — ● *0.7 g of disulfiram per kg alone.* Fig. *28: 0.7 g of disulfiram per kg followed 1 hour later by 1.6 g of methanol per kg.*

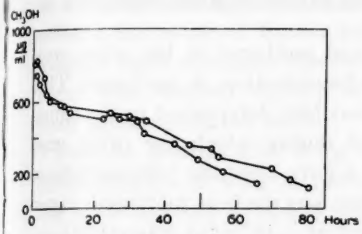

Fig. 29.

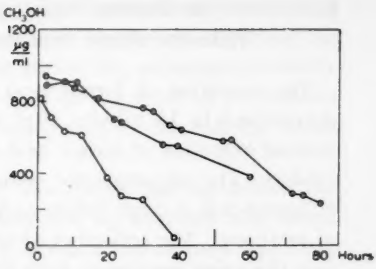

Fig. 30.

Fig. 29 and 30. — *Effect of disulfiram on the elimination from the blood of intravenously administered methanol.* Fig. *29: 1.0 g of disulfiram per kg followed 1 hour later by 0.8 g of methanol per kg.* Fig. *30:* ⊙—⊙ *1.0 g of disulfiram per kg, followed 12 hours later by 0.8 g of methanol per kg;* ○—○ *0.3 g of disulfiram per kg, followed 12 hours later by 0.8 g of methanol per kg.*

administered 1 hour earlier *per os*. During the first ten hours the elimination rate of methanol was nearly the same as without disulfiram, but after this time it was very greatly retarded and in some experiments was very near zero during some 10—20 hours. According to HALD, JACOBSEN and LARSEN (1949) the maximum effect of a single disulfiram dose on the ethanol metabolism begins 12—18 hours after the administration of disulfiram and the effect is very small during the first 5 hours. In the present experiments disulfiram was given 1 hour before methanol, so that the time which passed until the maximum effect of disulfiram on the methanol metabolism was reached corresponds very well with the data given by HALD *et al.* (1949) for ethanol metabolism.

When the disulfiram was given 12 hours before the intravenous administration of methanol (figure 30) the retarding effect was seen immediately after the administration of methanol, but otherwise the results were similar. If the dose of disulfiram was reduced to 0.3 g per kg no effect on the metabolism of methanol was seen.

All the blood samples taken in the experiments presented in this section were also analysed for the presence of formaldehyde. No formaldehyde, however, was detectable in any of the samples.

Excretion of Formic Acid and Methanol in the Urine of Rabbits after Administration of Methanol

The excretion of formic acid and methanol in the urine was determined in 13 rabbits after administration of methanol. The normal excretion of formic acid was first determined in the same rabbits. The experimental period during which the urine was collected was 3 days before and 3 days after the administration of methanol. The collection of urine was made in metabolic cages and the urine specimens were kept at —18° until determinations were made. The excretion is calculated in mg per 24 hours.

The mean normal urinary excretion of formic acid was in these rabbits 4.8 mg per 24 hours. The individual variations were great, the values ranging from 0.8 to 18.5 mg per 24 hours (table 20).

Table 20

Excretion of methanol and formic acid in the urine

Experiment No.	Dose of ethanol or disulfiram	Experimental period hours	Dose of methanol g per kg	Total dose of methanol g	Total excretion of methanol g	Excretion of formic acid mg per 24 hours	Normal excretion of formic acid mg per 24 hours
1		72	0.8 *per os*	1.98	0.20	12.4	9.2
2		72	0.8 *per os*	2.00	0.10	5.3	3.7
3		72	0.8 *per os*	1.92	0.15	14.1	18.5
4		72	0.8 iv.	2.28	0.19	0.7	1.0
5		72	1.6 *per os*	4.19	0.38	10.3	6.3
6	0.8 g per kg ethanol	72	0.8 *per os*	2.10	0.18	3.2	1.4
7	0.8 g per kg ethanol	72	0.8 *per os*	2.30	0.21	4.2	2.2
8	2.4 g per kg ethanol	72	0.8 *per os*	2.00	0.32	8.6	11.1
9	2.4 g per kg ethanol	72	0.8 *per os*	1.80	0.23	2.3	0.9
10	0.7 g per kg disulfiram	96	0.8 *per os*	2.12	0.48	1.1	1.7
11	0.7 g per kg disulfiram	96	0.8 *per os*	1.88	0.28	3.1	0.8
12	0.7 g per kg disulfiram	96	0.8 iv.	2.10	0.40	2.4	1.6
13	0.7 g per kg disulfiram	96	0.8 iv.	2.00	0.55	6.8	4.1

When methanol was administered in doses of 0.8—1.6 g per kg, no significant increase in the urinary excretion of formic acid was found (table 20, experiments 1—5). About 8 per cent of the administered methanol was excreted as such in the urine.

Effect of Ethanol and Disulfiram. — Administration of ethanol

to rabbits in doses of 0.8—2.4 g per kg simultaneously with 0.8 g of methanol per kg did not have any significant effect on the urinary excretion of formic acid (table 20, experiments 6—9). Slightly more methanol was excreted in the urine of the rabbits which also received ethanol, as compared to the rabbits which were given methanol alone.

Rabbits treated with 0.7 g of disulfiram per kg before the administration of 0.8 g of methanol per kg excreted also about the same amount of formic acid in the urine as they had done under normal conditions (table 20, experiments 10—13). However, the excretion of methanol in the urine was markedly increased, being about 25 per cent of the administered amount. The urine was collected during 4 days after the administration of disulfiram.

DISCUSSION

The elimination rate of methanol from the blood of rabbits in the present experiments was higher when the blood methanol concentration was high than when it was low. At concentrations less than about 800 μg of methanol per ml the deviation of the elimination curve from linearity was so small that when the fluctuations in the single values are also considered the curve can be treated as essentially linear.

There has been much disagreement in former investigations concerning the elimination of methanol from the blood. It was stated by BILDSTEN (1924), WIDMARK and BILDSTEN (1924) and BERNHARD and GOLDBERG (1933) that the elimination rate is constant and not proportional to the concentration of methanol in the blood. Linear elimination of methanol has been demonstrated also in dogs by NEYMARK (1936). The methanol elimination curves presented by NICLOUX and PLACET (1912) for dogs and rabbits were also practically linear. According to HAGGARD and GREENBERG (1939) the elimination rate of methanol is proportional to its concentration in the blood and also to the volume of pulmonary ventilation, and the elimination curve is correspondingly exponential. Exponential methanol elimination curves have been obtained for rabbits by AGNER and BELFRAGE

(1947), who used the specific chromotropic acid method for the determination of methanol.

Similar confusion is also to be seen in the literature concerning the elimination of ethanol (see e.g., JACOBSEN 1952 a, b, MARSHALL and FRITZ 1953). In the case of ethanol, however, the rectilinear elimination down to the lower limit of WIDMARK's method, 0.01 per cent, has been rather well established (NEYMARK and WIDMARK 1942, JACOBSEN 1952 a, b), and also the enzymic kinetics of pure horse liver alcohol dehydrogenase are in accord with the rectilinear elimination (THEORELL and BONNICHSEN 1951).

The elimination rate of methanol from the blood of rabbits was somewhat lower in the present study than in the experiments of AGNER and BELFRAGE (1947), the values being 0.00042 and 0.00060 mg per ml per minute respectively at the lower blood methanol level under 800 μg per ml, and 0.00120 and 0.00170 when the blood methanol concentration was 2 500—800 μg per ml. This difference, however, is not so great that it could not be explained on the basis of such possible factors as differences in the breed of the rabbits and in their diet and the general conditions. The rabbits used in the present study were rather old, being about $2\,^1/_2$ to 3 years of age, although in a good physical condition. The values obtained by BILDSTEN's (1924) bichromate method were still higher than those of AGNER and BELFRAGE (1947), being about 0.00070—0.00080 mg per g per minute (BILDSTEN 1924, WIDMARK and BILDSTEN 1924, BERNHARD and GOLDBERG 1933).

The administration of ethanol simultaneously with methanol or during its elimination clearly retarded the elimination of methanol from the blood in these experiments. This agrees well with the competitive inhibition of the utilization of methanol by ethanol which was observed in the liver and kidney homogenates.

When the dose of ethanol was 0.8 g per kg, its effect lasted about 4—5 hours. According to OLOW (1924) the elimination rate of ethanol from the blood is 0.0042 mg per g per minute in rabbits. If we use this constant and assume a similar distribution of methanol and ethanol in these rabbits, the administered 0.8 g of ethanol per kg was metabolized within 3—4 hours. When the

dose of ethanol was 2.0 g per kg, the retarding effect lasted for about 12 hours, and this amount of ethanol thus was metabolized in 8—10 hours. The retarding effect thus lasted approximately until the ethanol was metabolized, assuming that methanol does not greatly affect the oxidation of ethanol at these dosage levels. Calculations based on the results of the other experiments gave similar results. These results are well in accord with the isotope experiments of BARTLETT (1950 b) and with the experiments of LEAF and ZATMAN (1950) on the excretion of methanol in the urine after administration of ethanol and methanol.

The elimination of methanol from the blood of rabbits was also retarded in the experiments of AGNER and BELFRAGE (1946), in which they administered ethanol and methanol simultaneously to two rabbits.

The total excretion of methanol in the urine was slightly increased in the experiments in which also ethanol was given. This increase was due to the slower decrease in the blood methanol concentration in these cases. The absolute increase in the urinary excretion of methanol was, however, so small at these low dosage levels of ethanol that it had no significance in the elimination of methanol.

Disulfiram (tetraethylthiuram disulphide, antabuse) sensitizes human subjects to ethanol (HALD, JACOBSEN and LARSEN 1948) and it has been widely used in the therapy of chronic alcoholism. This sensitizing effect has been shown to be due to an increased acetaldehyde content in the blood after the intake of disulfiram and ethanol (HALD and JACOBSEN 1948, ASMUSSEN, HALD and LARSEN 1948). LARSEN (1948) studied also the effect of disulfiram on rabbits in combination with ethanol. He found five times as much acetaldehyde in the blood as was seen after the same dose of ethanol given to untreated rabbits. An increased blood acetaldehyde concentration after the intake of disulfiram and ethanol has later been found in all animal species investigated, including dog (NEWMAN and PETZOLD 1951), rat (GAL and GREENBERG 1954), pigeon (NOWINSKI and EWING 1953) and rabbit (HALD, JACOBSEN and LARSEN 1949, CARRATALÁ 1950, CZYZYK 1956, FUJIWARA 1953).

Although there are numerous studies on the effect of disulfiram

on the metabolism of ethanol *in vivo*, practically nothing is known of its effects on the metabolism of methanol. Disulfiram has been reported to increase the acute toxicity of methanol and formaldehyde to mice (GILGER, POTTS and JOHNSON 1952, OWE-LARSSON and SKOG 1951), which speaks in favour of a similar effect of disulfiram on the metabolism of methanol and ethanol.

If the effect of disulfiram on the metabolism of methanol were analogous to its effect on the metabolism of ethanol, there should occur an accumulation of formaldehyde in the blood of rabbits treated with disulfiram and methanol. No formaldehyde, however, could be demonstrated in such cases in the present investigation. However, formaldehyde is a very reactive compound and could have been partly bound to proteins or other substances or metabolized via other routes not sensitive to disulfiram. The oxidation of methanol to formaldehyde is also a very slow process, especially in disulfiram-treated rabbits, in comparison to the oxidation of ethanol, and therefore the formaldehyde possibly formed may have been totally metabolized via the possible disulfiram-insensitive routes.

Although no formaldehyde was accumulated in the present experiments in the blood of rabbits treated with disulfiram and methanol, disulfiram in doses of 0.7 g per kg or more retarded markedly the elimination of methanol from the blood. The elimination rate of methanol was near 0 for 10—20 hours in many experiments, and even this slight elimination might be entirely accounted for by excretion in the expired air and in the urine.

Moderate doses of disulfiram do not affect the elimination rate of ethanol in humans, dogs or rabbits (HALD *et al.* 1948, NEWMAN and PETZOLD 1951, LARSEN 1948, FUJIWARA 1953). However, the rate was retarded in some of the disulfiram-treated dogs (NEWMAN and PETZOLD 1951). It was demonstrated by HALD *et al.* (1949) that when the amount of disulfiram resorbed exceeded 1 gram the ethanol elimination rate was below normal in rabbits and could be as low as zero. Increasing amounts up to 1 gram of disulfiram resorbed by the animal resulted in increasing amounts of acetaldehyde in the blood. Although no determinations of the amount of resorbed disulfiram were made in the present experiments, the doses of 0.7 g per kg or more were

so high that more than 1 g of disulfiram should have been resorbed by the animals. Thus the effect of disulfiram on the elimination rate of methanol is similar to its effect on the elimination rate of ethanol.

The increase in the total urinary excretion of methanol when also disulfiram was given was due to the persistence of high blood methanol concentrations in these animals. Apparently also the total elimination of methanol in the expired air was higher in the case of disulfiram-treated rabbits than in controls.

The excretion of formic acid in the urine of rabbits after the administration of methanol showed in the present experiments no difference from the normal excretion, which also was rather low. This is in accordance with the earlier studies of BASTRUP (1947) and LUND (1948 a, b). The administration of ethanol or disulfiram had no effect in the present experiments on the urinary excretion of formic acid in the methanol-treated rabbits.

GENERAL DISCUSSION

Methanol can be metabolized in animal tissues *in vivo*, as well as *in vitro* in homogenates and slices, as is demonstrated in the present experiments. Formaldehyde is an intermediate in these reactions, and is partly accumulated in the reaction mixture in the *in vitro* experiments but *in vivo* is presumably metabolized as rapidly as it is formed.

The long delaying of methanol in the organism after administration, in contrast to ethanol, has been recognised for many years. A part of the ingested methanol is excreted as such in the expired air and the urine and the rest is slowly oxidized to formaldehyde and formic acid. These are oxidized further to carbon dioxide and water but the organism can also use them in synthetic reactions, as, for instance, in the formation of labile methyl groups and thus in the synthesis of methionine, choline, creatine, thymine and adrenaline. The carbon atom of formaldehyde or formic acid can also be incorporated into serine and purines. In addition to these one-carbon group reactions, formaldehyde has also other possibilities to react in the intermediary metabolism. It can condense with various substances and then be metabolized further, give rise to ribose, etc. The carbon atom of methanol thus has many different pathways in the metabolism. However, it is probable that the greater part of the administered methanol which is utilized in the organism is directly oxidized to carbon dioxide and water and that only a relatively small part is incorporated into other compounds (BARTLETT 1950 a).

It has been suggested that most of the ingested methanol is eliminated in the expired air and that oxidation in the organism plays only a minor rôle in the elimination of methanol (HAGGARD and GREENBERG 1939). However, it is very difficult to explain

the marked retardation of the elimination of methanol from the blood of rabbits treated with disulfiram or ethanol if the excretion via the lungs is regarded as the main elimination mechanism of methanol. The results of the experiments with isotope-labelled methanol are also against this view (BARTLETT 1950 a, HEVESY 1954). When the blood methanol concentration is high, the excretion of methanol in the expired air and in the urine may, however, be considerable and may partly be responsible for the greater elimination rate observed at the higher blood methanol concentrations.

We do not exactly know at present which enzymes are responsible for the oxidation of methanol to carbon dioxide and water *in vivo*. Both catalase and alcohol dehydrogenase are implicated in the oxidation of methanol to formaldehyde. Although catalase is capable of catalysing coupled oxidations of methanol *in vitro*, its participation to any great degree in the oxidation of methanol *in vivo* is not very likely. Crystalline horse liver alcohol dehydrogenase does not catalyse the oxidation of methanol (THEORELL and BONNICHSEN 1951), but less purified liver alcohol dehydrogenase preparations are able to oxidize methanol to formaldehyde (LUTWAK-MANN 1938, ZATMAN 1946, KOIVUSALO unpublished observations). Partially purified alcohol dehydrogenase preparations from horse liver can also reduce diphosphopyridine nucleotide in the presence of methanol, although at only one-eighth of the rate of this reaction in the presence of ethanol (KOIVUSALO unpublished observations).

The oxidation of formaldehyde can be catalysed *in vivo* by the different aldehyde oxidizing enzymes, possibly for the greater part by the specific formaldehyde dehydrogenase (STRITTMATTER and BALL 1955). It also may first be condensed with other substances and then oxidized. The direct oxidation, however, seems more likely *in vivo*. In the present experiments in which formaldehyde was readily utilized in animal tissue homogenates, disulfiram caused only a slight inhibition of this utilization. This speaks somewhat against the participation in this reaction of the aldehyde-oxidizing enzymes such as xanthine oxidase, aldehyde dehydrogenase, aldehyde oxidase and d-glyceraldehyde-3-phosphate dehydrogenase, which are all inhibited by disulfiram

(KJELDGAARD 1949, RICHERT, VANDERLINDE and WESTERFELD 1950, GRAHAM 1951, NYGAARD and SUMNER 1952). We have no knowledge of the sensitivity of the specific formaldehyde dehydrogenase to disulfiram. Neither did the administration of disulfiram cause any accumulation of formaldehyde in the blood after the intake of methanol in the *in vivo* experiments.

Formic acid formed from the formaldehyde is then oxidized to carbon dioxide and water by coupled oxidations catalysed by catalase or by a specific formicodehydrogenase. The existence of the latter enzyme in the animal organism is not certain, however.

In the present experiments, ethanol inhibited competitively the utilization of methanol in liver and kidney homogenates and retarded the elimination of administered methanol from the blood of rabbits. These results are readily explained if we assume that alcohol dehydrogenase is responsible for the oxidation of methanol and that the affinity of ethanol to the enzyme is much greater than that of methanol. In the case of catalase the explanation is difficult, unless we assume that ethanol inhibits the enzymes forming hydrogen peroxide. Disulfiram inhibited also the utilization of methanol in liver homogenates and retarded its elimination from the blood in rabbits. In this case a general metabolic inhibition may also be responsible for the effect of disulfiram. Crystalline alcohol dehydrogenase is not inhibited by disulfiram (KJELDGAARD 1949), but many of the flavoproteins which can function as aerobic oxidases and form hydrogen peroxide are inhibited by disulfiram.

There is at present no evidence that methanol might occur as an intermediate innormal metabolism. The small amounts of methanol which have been recovered from normal human urine are probably derived from the pectins in the food, which may contain up to 12 per cent methanol (VON FELLENBERG 1917, JANSCH 1924). On the other hand, formaldehyde or its near derivatives can be regarded as normal metabolites in the animal organism.

The purpose of this investigation has been to give a general picture of the metabolism of methanol and formaldehyde in the animal organism both *in vivo* and *in vitro*. To obtain detailed information on the individual enzymes which catalyse these reactions further studies are required, using purified enzyme systems and methanol and formaldehyde as substrates.

SUMMARY

The purpose of this investigation was to study the metabolism of methanol and formaldehyde in the animal organism. In the *in vitro* experiments the utilization of methanol and accumulation of formaldehyde were studied in rat and guinea pig tissue preparations incubated aerobically with methanol at 37°. The optimum conditions for these reactions were determined and the effect of added metabolic cofactors and inhibitors was investigated. The effect of ethanol and disulfiram was also studied. Most of the experiments were made with liver homogenates, but tissues other than liver were also used. Experiments were also made with tissue slices. The utilization of added formaldehyde was investigated in tissue homogenates. In the *in vivo* experiments the elimination of administered methanol from the blood was followed in normal rabbits and in rabbits treated with ethanol or disulfiram.

The determinations of methanol and formaldehyde were made by photometric methods based on the chromotropic acid reaction, and a method for the simultaneous determination of formaldehyde and methanol in the reaction mixtures was worked out.

Rat and guinea pig liver homogenates readily utilized methanol when incubated aerobically at 37°. Of the methanol utilized during any period of time 5—20 per cent was recovered from the reaction mixture as formaldehyde. No effect was seen when boiled homogenates were used. The utilization of methanol was most rapid during the first 30 minutes of incubation and also the accumulation of formaldehyde was then greatest. Later, when less methanol was utilized, the amount of accumulated formaldehyde gradually diminished. The optimum pH range for the aerobic utilization of methanol in liver homogenates was 7. 3—8. 3 and the accumulation of formaldehyde was parallel with the utilization of methanol.

Only small amounts of methanol were utilized anaerobically and no formaldehyde was then accumulated. Ageing of the homogenate at +2° for 12 hours increased the accumulation of formaldehyde without causing any difference in the utilization of methanol. Storage at −18° for 48 hours had no effect.

Liver tissue homogenates were most active in the utilization of methanol. Homogenates from kidney, spleen, lung, testis and heart muscle tissues also utilized methanol, the activity diminishing in the above order. Homogenates from skeletal muscle and brain showed no activity. Guinea pig tissues utilized generally less methanol than rat tissues but no qualitative differences between them was noted.

The utilization of methanol in liver homogenates was inhibited 73 per cent by 0.01 M monoiodoacetate and the accumulation of formaldehyde diminished by 43 per cent. Corresponding values for 0.050 M malonate were 83 and 100 per cent, and for 0.005 M cyanide 90 and 100 per cent. Disulfiram, 0.25 mg per ml, inhibited the utilization of methanol in liver homogenates by about 60 per cent, and the accumulation of formaldehyde diminished by about 20 per cent.

Added diphosphopyridine nucleotide, adenosine triphosphoric acid, diphosphothiamine, reduced glutathione, cytochrome *c* and Mg^{++} ions had no clear effect on the utilization of methanol and accumulation of formaldehyde in liver homogenates. In homogenates from lung and heart muscle tissues the addition of adenosine triphosphoric acid and diphosphopyridine nucleotide together increased both the utilization of methanol and the accumulation of formaldehyde. The addition of pteroylglutamic acid and adenosine triphosphoric acid together decreased the accumulation of formaldehyde from methanol in liver homogenates. The addition of vitamin B_{12} had no effect.

Ethanol inhibited competitively the utilization of methanol in liver and kidney homogenates and diminished also the accumulation of formaldehyde. The inhibition was seen when the molar ratio of ethanol to methanol was 1: 2 or more.

Liver slices utilized methanol at about the same rate as liver homogenates. The accumulation of formaldehyde in the slices was smaller than in homogenates.

Homogenates from liver, kidney and lung tissues utilized added formaldehyde, the activity decreasing in the above order. Disulfiram had only a slight inhibitory effect on the utilization of formaldehyde in liver homogenates. Added ethanol had no effect on the utilization of formaldehyde in liver homogenates. Added methanol diminished the utilization of added formaldehyde, and *vice versa*.

The elimination of methanol administered perorally and intravenously in doses of 0.2—3.4 g per kg was followed in rabbits. The elimination rate of methanol from blood was about 0.00042 mg per ml per minute at blood methanol concentrations of less than about 800 μg per ml, and about 0.00120 at blood methanol concentrations of 2 500—800 μg per ml. When ethanol was administered simultaneously with methanol or during its elimination, the elimination of the latter from the blood was retarded for a time roughly corresponding to that required for the metabolization of the administered ethanol.

Disulfiram administered perorally 1—12 hours before methanol in doses of 0.7—1.0 g per kg retarded distinctly the elimination of methanol from the blood. No effect was seen after 0.3 g of disulfiram per kg.

No formaldehyde was detected in the blood of rabbits administered either methanol, methanol and ethanol, methanol and disulfiram, or disulfiram alone.

The administration of methanol in the above doses did not increase the excretion of formic acid in the urine. About 8 per cent of the administered methanol was recovered from the urine. Administration of ethanol or disulfiram in addition to methanol increased the excretion of methanol in the urine. They had no effect on the excretion of formic acid.

The results obtained and their correlations as well as the metabolism of methanol and formaldehyde in the animal organism in general are discussed.

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